APPENDIX 1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Asgeir Sæbo *et al.*		
Serial No.:	09/438,104	Group No.:	1614
Filed:	November 10, 1999	Examiner:	Jones, D.
Entitled:	**Conjugated Linoleic Acid Compositions**		

Declaration of Asgeir Sæbo

Assistant Commissioner for Patents
Washington, D.C. 20231

CERTIFICATE OF MAILING UNDER 37 C.F.R. §1.8(a) (1)(i)(A)

I hereby certify that this correspondence (along with any referred to as being attached or enclosed) is on the date shown below, being deposited with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

Dated: 11-25-03 By: Susan M. McClintock
Susan M. McClintock

I, Asgeir Sæbo, state as follows:

1. My present position is Vice President of Technology, Natural ASA.

2. I have reviewed the above captioned patent application, of which I am an inventor, the Office Action mailed April 10, 2002, and the Cain and Pariza references cited as prior art.

3. In the repeat of Cain, the conjugation conditions were the same as those described in Example 6 of WO97/18320. The results of the conjugation reactions were analyzed by GC-MS. The results are attached at Tab 1. As can be seen, this conjugation method resulted in a conjugated linoleic acid composition comprising approximately 3.49% c11,t13 CLA and 2.24% t9,t11and t10,t12 CLA. The t8,c10 isomer co-elutes with the c9,t11 isomers, but almost always occurs in a one to one proportion to the c11,t13 isomer. I note that this method is very similar to the method utilized in the Sugano reference, which was discussed in my previous Declaration. My work confirms that these methods produce CLA with relatively high levels of undesirable isomers.

4. The Examiner states at page 4 of the Office Action that Cain teaches CLA compositions

that are composed of 49.7% c9,t11 and 50.3% t10,c12 CLA, and that because these numbers add up to 100% no other isomers were present. However, the percentages reported do not mean that the other isomers were not present, as was found in my repeat of Cain. This discrepancy is explainable by the facts that 1) methods for the analysis of CLA compositions in 1996 were rather crude and 2) Cain may have simply chosen not to include non-active isomers when reporting their results. Improved methods for detecting the various isomers of CLA were not developed until well after the 1995 priority date of Cain. This fact is substantiated by Yurawecz *et al.* (attached at Tab 2), who state "the CLA products analyzed in this study were found to contain up to 12 geometric and positional CLA isomers. These findings are based on appropriate and improved analytical methodologies [including gas chromatography techniques] that have only recently been developed." (Yurawecz, *p.* 281). Thus, Cain et al. may not have conducted an analysis that could detect the isomers in questions. Consideration of Example 18 of Cain et al. supports this analysis. The inventors state that their compositions, produced by the method of Example 6, contained 63.8% CLA, of which 48.9% was the cis 9, trans 10 isomer and 51.1% was the trans 10, cis 12 isomer. This means that the inventors provide no analysis of the remaining 36.2% of their composition. The 8,10; 11,13; and trans-trans isomers that are discriminated against in the present invention and detected in my repeat of Cain could well have been present in this fraction.

5. I further note that the formation of the c11,t13 isomer from the t10,c12 isomer and the t8,c10 isomer from the c9,t11 isomer is caused by a process known as thermal sigmatropic rearrangement. This process is described in Chapter 5 of the book Advances in *Conjugated Linoleic Acid Research, Volume 2*, J. Sebedio, W.W. Christie, and R. Adolf, Eds., AOCS Press, Champaign, IL, 2002. I wrote this chapter. Briefly, this research described in this chapter establishes that the formation of the 8,10 and 11,13 isomers is a necessary consequence of heating compositions containing the t10,c12 and c9,t11 isomers. Thus, whenever compositions containing t10,c12 and c9,t11 CLA are heated at temperatures such as those used by Cain et al. (i.e., 180°C for about 2 - 2.5 hours), 8,10 and 11,13 isomers are necessarily produced. Because Cain et al. does not describe the presence of these isomers in their compositions, the only reasonable conclusion is that they did not analyze for these isomers or chose to delete these isomers from their report because they were not considered to be active isomers.

6. With regard to the rejection over Pariza (U.S. Pat. No. 5,856,149), I note that Pariza et al. does not teach compositions that contain both c9,t11 and t10,c12 isomers while containing less than 1% 8,10 and 11,13 isomers.

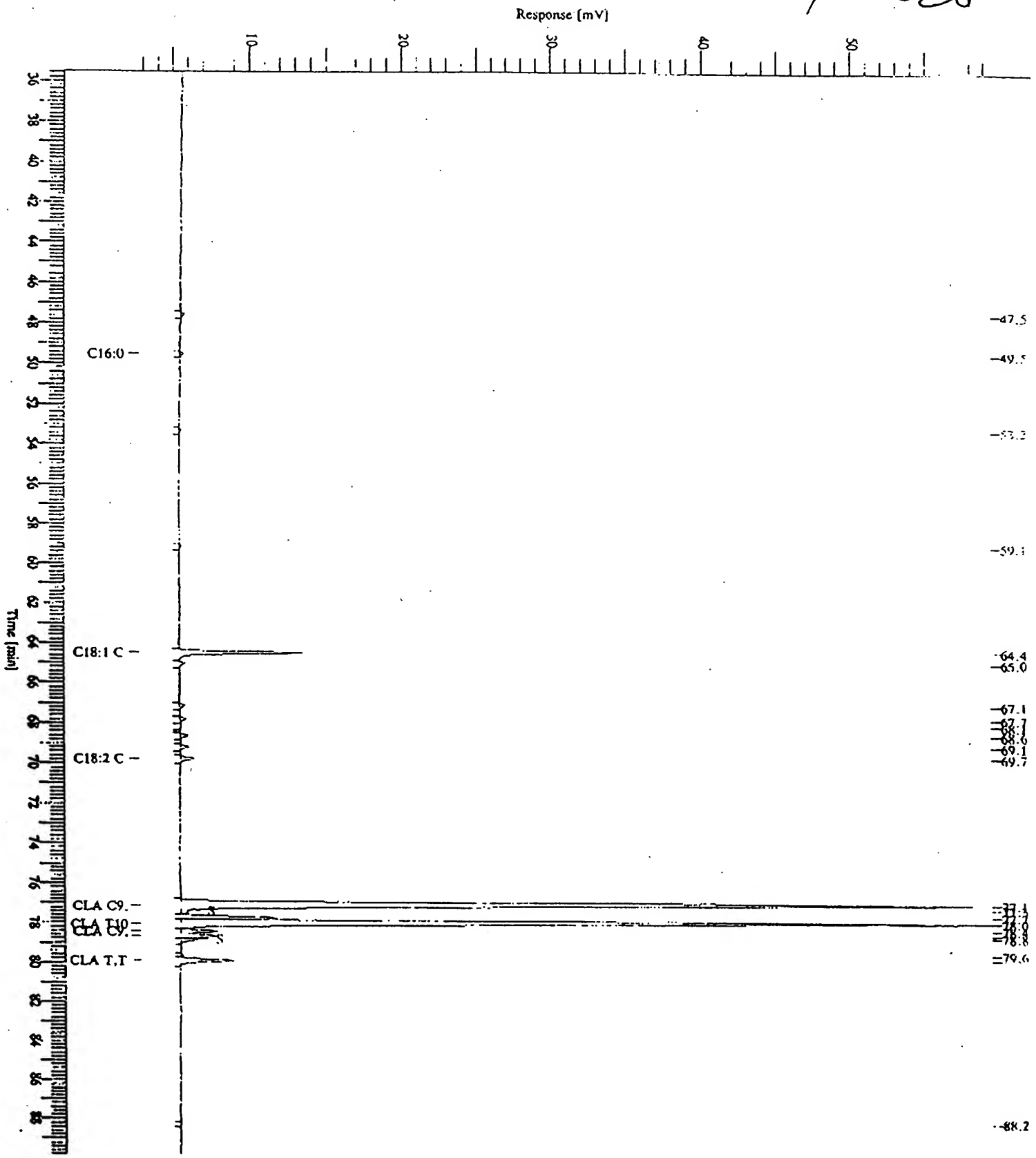
7. I further declare that all statement made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Asgeir Sæbo

Date: Oct 31 2003

Sample Name : 6659: A01348, 024/96-1, CLA FPA Sample #: 001 Page 1 of 1
FileName : D:\TCWS Data\data\Data 100E 1000-15[illegible]100e1001.raw
Date : 19.11.01 11:48:38
Method : Time of Injection: 19.11.01 09:08:32
Start Time : 35.53 min End Time : 89.83 min Low Point : 2.75 mV High Point : 59.51 mV
Plot Offset: 2.75 mV Plot Scale: 56.8 mV

WO 97/18320



Software Version : 6.1.2.0.1:D19
Sample Name : 6659: A01348, 024/96-1, CLA FFA
Instrument Name : GC
Rack/Vial : 0/1
Sample Amount : 1.000000
Cycle : 1
Date : 19.11.01 11:48:37
Data Acquisition Time : 19.11.01 09:08:32
Channel : B
Operator : Operatør
Dilution Factor : 1.000000

Result File : D:\TCWS Data\data\Data 100E 1000-1999\100e1001.rst
Sequence File : D:\TCWS Data\sekvenser\100E.20.10.00..seq

FATTY ACID PROFILE REPORT

PERKIN ELMER AUTOSYSTEM XL GC

Column: WCOT FUSED SILICA 100 m x 0.25 mm COATING CP-SIL 88 DF= 0.2 Chrompack cat.no: 7489
Carrier Gas: He, 30.0 PSI
Method: 100E.mth
Temp: 80 C (2 min)->45 C/ min->130 C (0 min)->1 C/ min-> 220 C (10 min)
Injection: Splitless, 240 C
Detector: FID, 280 C

Peak #	Time [min]	Component Name	Area [%]	Area [µV·s]	Height [µV]
1	47.557		0.14	2040.57	221.66
2	49.507	C16:0	0.12	1770.08	234.26
3	53.277		0.07	1043.10	118.41
4	59.139		0.07	1079.52	131.55
5	64.461	C18:1 c9	4.84	72109.91	8053.81
6	65.035		0.23	3435.33	396.61
7	67.125		0.25	3718.15	401.86
8	67.795		0.28	4195.57	459.60
10	68.621		0.31	4688.64	520.82
11	69.176		0.33	4880.16	532.98
12	69.744	C18:2 c9,c12	0.53	7977.36	868.60
13	77.128	CLA c9,t11+t8,c10	42.84	638739.60	52812.75
14	77.371		0.28	4120.52	216.07
15	77.752	CLA c11,t13	3.49	51987.22	6233.41
16	78.067	CLA t10,c12	40.35	601682.23	54289.00
17	78.437	CLA c9,c11	1.36	20327.77	2373.19
18	78.664	CLA c10,c12	1.61	24007.50	2280.68
19	78.808		0.58	8661.37	1107.38
20	79.693		0.08	1265.48	173.63
21	79.909	CLA t,t 9,11+10,12	2.24	33420.59	3512.11
			100.00	1491150.67	134938.38

Missing Component Report

Component	Expected Retention (Calibration File)
C18:0	0.001

19.11.01 11:48:37 Result: D:\T\ /S Data\data\Data 100E 1000-1999\100e1001.rst

Analyzed by: Natural ASA, Hovdebygda

Approved by: _____

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Inhalt

Contents

Variations in isomer distribution in commercially available conjugated linoleic acid*

Martin P. Yurawecz[1], Najibullah Sehat[1], Magdi M. Mossoba[1], John A.G. Roach[1], John K. G. Kramer[2], and Youh Ku[1]

Conjugated linoleic acid (CLA) has been reported to have anticarcinogenic and antiatherogenic properties, to repartition body fat, to build bone mass, to normalize glucose tolerance, and to reduce hyperglycemia and diabetes. CLA products are now commercially available, and there is considerable interest in studying CLA because of this range of reported beneficial effects. However, little is known about the composition of these preparations. Representative commercial CLA products in capsule or liquid (aqueous or oily) form were analyzed for their CLA content and isomer composition using gas chromatography (GC), silver ion-high performance liquid chromatography (Ag^+-HPLC) and spectroscopic techniques. The content of CLA in the preparations varied widely. Based on the GC-internal standard technique, total CLA varied from 20 to 89% by total weight and 28 to 94% of total fat. One product contained no CLA. The isomer distributions were generally of two types: those with two major CLA positional isomers, and those with four major CLA positional isomers. All the CLA preparations in capsule form contained the four isomer mixture, while the liquid preparations contained from two to four CLA positional isomers.

Unterschiede in der Isomerenverteilung kommerziell erhältlicher konjugierter Linolsäure. Von konjugierter Linolsäure (CLA) wurde berichtet, daß sie anticanzerogene und antiatherogene Eigenschaften hat, Körperfett repartioniert, Knochenmasse aufbaut, Glukosetoleranz normalisiert und Hyperglykämie und Diabetes reduziert. CLA-Produkte sind jetzt kommerziell erhältlich, und es gibt wegen der oben aufgeführten prositiven Effekte ein beträchtliches Interesse daran, CLA zu studieren. Allerdings ist wenig bekannt über die Zusammensetzung dieser Herstellungen. Repräsentative kommerzielle CLA-Produkte in Kapsel- oder flüssiger Form (auf Wasser- oder Ölbasis) wurden mit Hilfe eines Gaschromatographen (GC), Silberionen-Hochdruckflüssigkeitschromatographie (Ag^+-HPLC) und spektroskopischer Techniken auf ihren CLA-Inhalt und ihre isomere Zusammensetzung analysiert. Der Inhalt der CLA in den Herstellungen variierte stark. Auf der Basis der GC-internen Standardtechnik schwankten die gesamten CLA zwischen 20 und 89% bezogen auf das Gesamtgewicht und zwischen 28 und 94% bezogen auf den Gesamtfettanteil. Ein Produkt enthielt keine CLA. Die Isomerverteilungen untergliederten sich allgemein in zwei Typen: solche mit zwei Positionsisomeren und solche mit vier Positionsisomeren. Alle CLA-Herstellungen in Kapselform beinhalteten das Gemisch der vier Isomere, während die flüssigen Herstellungen zwischen zwei und vier der Positionsisomere enthielten.

1 Introduction

Conjugated linoleic acid (CLA) has been reported to provide direct [1] or indirect [2] protection against several types of cancer, atherosclerosis [3, 4], and diabetes [5]. CLA has also been reported to improve feed efficiency [6] and increase muscle [7, 8], and bone mass [9]. These results were generally obtained in experimental animals fed commercial CLA preparations containing approximately equal amounts of four *cis/trans* conjugated octadecadienoic (18:2) acids: 8 *trans*, 10 *cis*-18:2; 9 *cis*, 11 *trans*-18:2; 10 *trans*, 12 *cis*-18:2; 11 *cis*, 13 *trans*-18:2; and minor amounts of the corresponding *cis,cis* and *trans,trans* CLA isomers [10]. Thus, the contribution(s) of the specific isomers to the observed effects are not known. In contrast, natural products, such as milk, cheese, and meat from ruminant animals contain mainly rumenic acid (9 *cis*, 11 *trans*-18:2) [11–13] with minor amounts of 7 *trans*, 9 *cis*-18:2 [14] and other isomers [15–17]. The total CLA content in these natural products ranges from trace to 2% of total fatty acids [12, 18, 19].

The present study was undertaken to determine the content and distribution of CLA isomers in commercially available CLA capsules and liquid products with labels stating to contain CLA. The CLA isomers were analyzed by gas chromatography (GC) and silver ion-high performance liquid chromatography (Ag^+-HPLC) as their fatty acid methyl esters (FAME), and identified by GC-electron ionization mass spectroscopy (GC-EIMS) and GC-direct deposition-Fourier transform infrared (GC-DD-FTIR) spectroscopy as their 4,4-dimethyloxazoline derivatives [10, 14, 17, 20].

2 Materials and Methods

2.1 Chemicals

Representative CLA preparations were purchased locally, from specialty chemical companies, or from the World Wide Web. Several pure CLA isomers were obtained as their free fatty acids from *Matreya*, Inc. (Pleasant Gap, PA). Acetonitrile and hexane were UV grade. Other solvents were distilled-in-glass quality. 2-Amino-2-methyl-1-propanol (95%) was purchased from *Aldrich Chemical Company*, Inc. (Milwaukee, WI). A 10% solution of trimethylsilyldiazomethane in hexane was obtained from *TCI America* (Portland, OR).

[1] US Food and Drug Administration, Center for Food Safety and Applied Nutrition, USA.
[2] Southern Crop Protection, Food Research Center, Agriculture and Agri-Food Canada, Canada.
* Presented in part at the 52nd International Deutsche Gesellschaft für Fettwissenschaft (DGF) Congress in Magdeburg, Germany, Sept. 13–15, 1998.

Anhydrous $NaOCH_3$/methanol was purchased from *Supelco*, Inc. (Bellefonte, PA.).

2.2 Lipid extraction

A known weight (approximately 25 mg) of each product was dissolved in 2 ml 1 N KOH in ethanol (95%) and hydrolyzed overnight in the dark at room temperature. For quantitative analyses, one mg of eicosanoic acid (23:0) was added as an internal standard. After hydrolysis, 5 ml of H_2O and one ml of 6 N HCl were added and the free fatty acids were extracted three times with 5 ml diethyl ether/petroleum ether (1:1). The combined extracts were washed with H_2O and dried over anhydrous Na_2SO_4, and the solvents removed under a stream of argon. Aqueous CLA samples were first extracted with petroleum ether/diethyl ether (1:1), and 25 mg of the extracted lipids were treated as described above.

2.3 Derivatizations

FAMEs were prepared for GC by dissolving the free fatty acids in one ml of benzene/methanol (4:1) to which 0.5 ml of a 10% solution of trimethylsilyldiazomethane in hexane were added [21]. The reaction was allowed to stand for 0.5 h with occasional gentle shaking. Thereafter, five drops of glacial acetic acid were added with gentle shaking. The same amount of glacial acetic acid was added to each of the solutions to destroy excess yellow trimethylsilyldiazomethane. Some solutions did not become clear on addition of glacial acetic acid. Then 5 ml of H_2O were added, and the reaction mixture was extracted with one ml of isooctane. The extract was subsequently dried over anhydrous Na_2SO_4.

The 4,4-dimethyloxazoline (DMOX) derivatives were prepared to determine the double bond position of CLA isomers. Ten to 20 mg of the free fatty acid product prepared above was added to a screw cap reaction tube (1 ml) and a threefold excess (w/w) of 2-amino-2-methyl-1-propanol was added. The tube was purged with argon, capped, and heated at 170 °C for 0.5 h in an oven. DMOX derivatives were then partitioned into petroleum ether as described previously [22].

2.4 Gas chromatography

The analyses of the FAMEs were carried out using a *Hewlett-Packard* (Palo Alto, CA) model 5890 gas chromatograph fitted with a flame-ionization detector. A CP-Sil 88 fused-silica capillary column (100 m × 0.25 mm i.d. × 0.2 µm film thickness; *Chrompack*, Bridgewater, NJ) was used, and H_2 was the carrier gas at a split ratio of 50:1. The column was operated at 75 °C for 2 min, then temperature-programmed at 5 °C/min to 185 °C, held for 30 min, followed by a second temperature program at 4 °C/min to 225 °C and held there for 33 min.

2.5 Ag^+-HPLC

The HPLC (*Waters 510* solvent delivery system; *Waters Associates*, Milford, MA) was equipped with an autosampler and 200-µl injection loops (*Waters 717*), a UV detector operated at 233 nm (*Waters 486* tunable absorbance), and a data system (*Waters Millennium*™ version 2.15). A *ChromSpher 5 Lipids* analytical silver impregnated column (4.6 mm i.d. × 250 mm stainless steel; 5 µm particle size; *Chrompack*, Bridgewater, NJ) was operated at room temperature. The mobile phase was 0.1% acetonitrile in hexane and operated isocratically at a flow rate of 1.0 ml/min. The retention times varied slightly between runs due to the insolubility of acetonitrile in hexane. However, these changes did not affect the relative elution sequence of CLA isomers. Typical injection volumes were 5–15 µl at a concentration of 1 mg total FAME per ml.

2.6 Gas chromatography – electron ionization mass spectrometry

The GC-EIMS analyses were performed by using a *Hewlett-Packard* (model 5890, series II) GC coupled to a mass spectrometer (*Autospec Q* mass spectrometer) and a data system (*OPUS 4000; Micromass*, Manchester, UK). The GC-EIMS system utilized version 2.1 BX software. This system was used with a 50 or 100 m CP-Sil 88 fused-silica capillary column. The GC-EIMS conditions were: splitless injection with helium or hydrogen as the carrier gas and sweep was restored 1 min after injection. The injector and transfer lines temperatures were 220 °C. The column was operated at 75 °C for one min after injection, then temperature-programmed 20 °C/min to 185 °C, held there for 15 min, then temperature-programmed 4 °C/min to 220 °C, and held there for 45 min.

Tab. 1. Conjugated linoleic acid (CLA) methyl ester isomers, as % of total CLA, in 13 commercial CLA preparations as determined by silver ion-high performance liquid chromatography (Ag^+-HPLC).

Product	*trans,trans*				*cis/trans*[a]				*cis,cis*			
	11,13	10,12	9,11	8,10	11,13	10,12	9,11	8,10	11,13	10,12	9,11	8,10
1 aqueous	0[b]	0	0	0	0	0	0	0	0	0	0	0
2 oil	0	1.1	1.0	tr[c]	0	47.1	50.8	tr	0	tr	tr	0
3 oil	0	0.5	0.5	0	1.1	50.2	47.6	tr	0	tr	tr	0
4 oil	0	1.1	1.3	0	0	45.8	50.7	tr	0	1.1	0.1	tr
5 oil	0	0.6	0.6	0	0	54.0	43.5	0	0	0.7	0.6	0
6 oil	1.5	5.4	11.9	7.1	2.2	38.3	21.7	tr	0	2.3	6.2	3.5
7 capsule	0.7	2.7	2.8	0.5	19.0	32.1	25.6	15.6	0.7	0.4	tr	tr
8 capsule	0.8	2.7	2.5	0.4	16.8	33.9	27.1	14.2	tr	1.7	tr	tr
9 capsule	1.0	3.1	2.8	0.5	16.9	33.7	26.9	14.0	0.6	0.5	tr	tr
10 capsule	0.7	2.5	2.5	0.6	15.5	31.0	27.7	14.2	0.8	2.2	1.8	0.6
11 capsule	0.4	2.8	2.9	0.5	14.4	29.1	30.0	15.9	0.5	1.5	2.1	tr
12 capsule	1.3	3.3	3.4	1.1	19.8	25.9	21.6	16.5	1.2	2.7	2.4	1.0
13 oil	4.0	5.4	5.4	1.7	19.7	26.8	25.6	10.5	0.2	0.1	0.6	0.1

[a] The CLA isomers exist either in the *cis,trans* or *trans,cis* configuration. [b] 0, not detectable. [c] tr, trace (<0.05%).

2.7 GC-direct deposition Fourier transform infrared spectroscopy

GC-DD-FTIR was performed using a *Bio-Rad* (Cambridge, MA) *Tracer™ GC-FTIR 60A* spectrometer system. This system was used with a 50 m CP-Sil-88 fused-silica capillary column as described previously [23, 24].

3 Results and Discussion

Preparations of CLA were capsules or liquids that were water-based or oil-based; some contained non-lipid material. Their chemical compositions were not known. Based on the assumption that the CLA products consisted of esters, free fatty acids or combinations thereof, all products were hydrolyzed under alkali conditions and subsequently methylated by using trimethylsilyldiazomethane as catalyst to ensure preservation of the original distribution of CLA isomers [25].

Total fatty acid compositions of the 13 CLA products were determined. The internal standard (23:0) added to the CLA products provided a mean to determine the amount of total fatty acids in the original CLA mixture. When this total fatty acid value was compared to the 25 mg of starting material used, an approximate estimate of the non-lipids in the sample was obtained. The approximate amount of non-lipid material calculated for these products ranged from 3 to 28%. In addition, unidentified FAMEs ranged from 1 to 29%. The

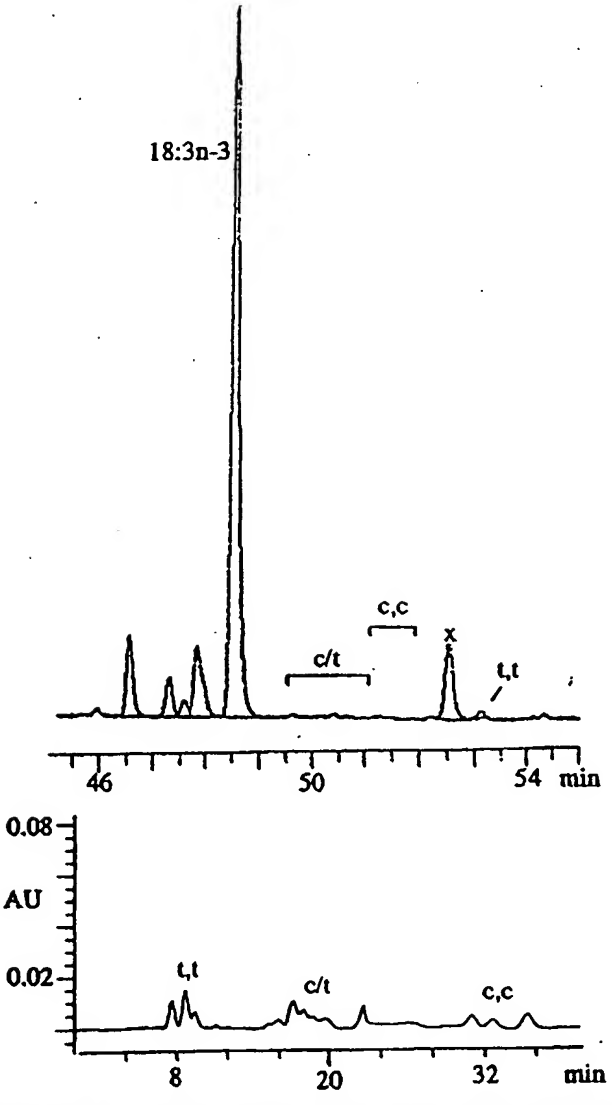

Fig. 1. Gas (upper graph) and silver ion-high performance liquid (lower graph) chromatograms of a commercial conjugated linoleic acid (CLA) preparation containing no CLA. The corresponding CLA regions in each chromatogram are labelled; x is an unknown component. The absorbance scale is shown in the lower graph to indicate the low response found in the CLA region.

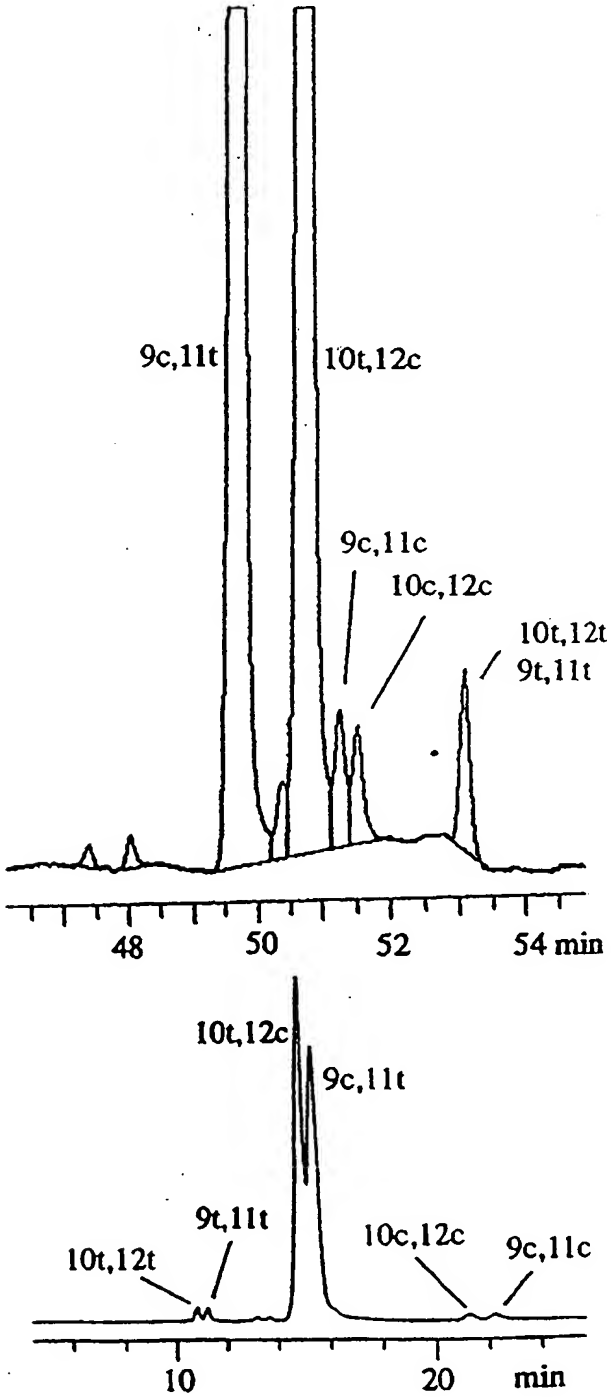

Fig. 2. Gas (upper graph) and silver ion-high performance liquid (lower graph) chromatograms of a representative commercial conjugated linoleic acid (CLA) preparation consisting primarily of two CLA isomers, 9 *cis*, 11 *trans*-18:2 and 10 *trans*, 12 *cis*-18:2. Peaks corresponding to CLA isomers in each chromatogram are labelled.

major fatty acids, other than the CLA isomers found in the preparations, included palmitic (16:0), stearic (18:0), oleic (18:1n-9), and linoleic (18:2n-6) acids. Their combined content ranged from 1 to 84% in the products examined. Based on the GC-internal standard technique, total CLA content ranged from 0 to 94% of the total FAMEs, or 0 to 89% of the mass content of the products.

The CLA isomer distributions in the products, analyzed by a combination of GC and Ag^+-HPLC, are shown in Tab. 1. No CLA was found in product 1. The CLA-containing products fell into two catagories: those composed of two major CLA positional isomers (9 *cis*, 11 *trans*-18:2 and 10 *trans*, 12 *cis*-18:2), and those composed of four major CLA positional isomers (8 *trans*, 10 *cis*-18:2; 9 *cis*, 11 *trans*-18:2; 10 *trans*, 12 *cis*-18:2, and 11 *cis*, 13 *trans*-18:2). Other minor CLA isomers were present at much lower concentrations, but are not reported. All the CLA products in capsule form contained the mixture of four isomers, while the liquid products contained either two or four CLA positional isomers. Representative GC and Ag^+-HPLC chromatograms for these groups are shown in Figs. 1, 2, and 3, respectively.

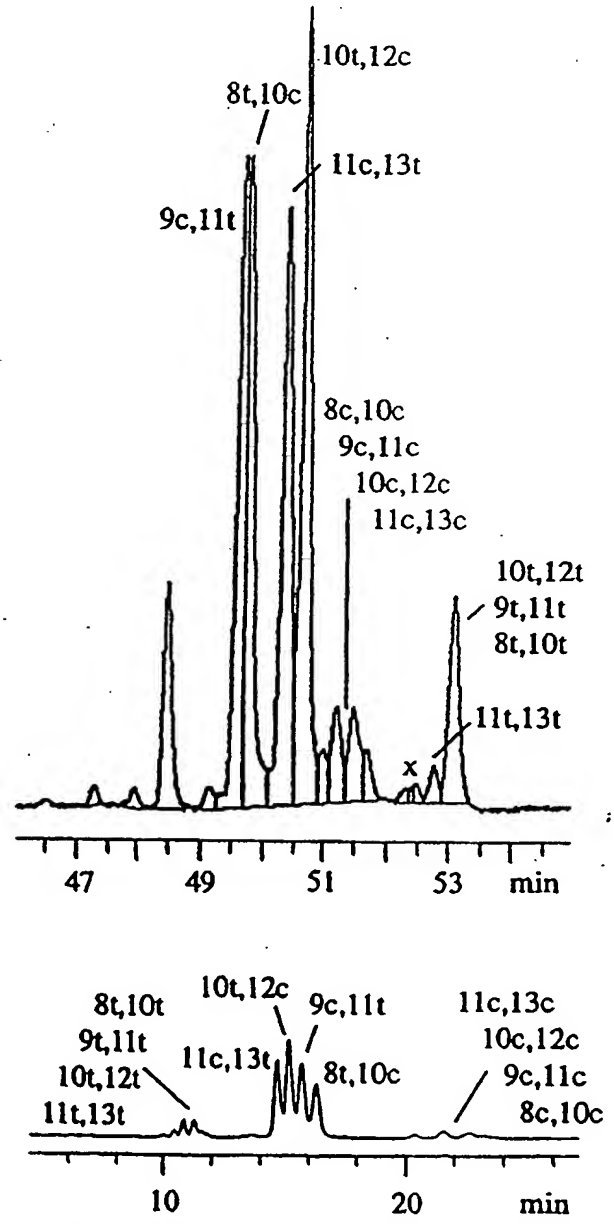

Fig. 3. Gas (upper graph) and silver ion-high performance liquid (lower graph) chromatograms of a representative commercial conjugated linoleic acid (CLA) preparation consisting primarily of four CLA isomers, 8 *trans*, 10 *cis*-18:2, 9 *cis*, 11 *trans*-18:2, 10 *trans*, 12 *cis*-18:2 and 11 *cis*, 13 *trans*-18:2. Peaks corresponding to CLA isomers in each chromatogram are labelled; x is an unknown component.

An explanation for the differences in isomer distributions among the products was not available. Alkali isomerization of 18:2 n-6 in laboratory scale batches prepared according to published procedures [12, 26, 27] resulted in the formation of only two CLA isomers, i.e., 9 *cis*, 11 *trans*-18:2 and 10 *trans*, 12 *cis*-18:2. Alkali isomerization under large-scale, and possibly more severe conditions, may have produced the four CLA positional isomer pattern observed in the commercial CLA preparations described here. This will need to be confirmed.

The GC analyses were based on use of a 100 m polar capillary column. In this system, 8 *trans*, 10 *cis*-18:2 was not resolved from 9 *cis*, 11 *trans*-18:2. A shoulder or a split peak may occasionally be evident when the amounts of these two isomers are approximately equal. In contrast, 11 *cis*, 13 *trans*-18:2 eluted before and was resolved from 10 *trans*, 12 *cis*-18:2 using this GC column (Fig. 3, upper graph). The *cis,cis* CLA isomers eluted after 10 *trans*, 12 *cis*-18:2 in the order 8, 10-, 9, 11-, 10, 12-, and 11,13-18:2 as established previously [10, 20]. The last CLA isomers to elute were the *trans,trans*, consisting of a small peak due to 11,13-18:2 followed by an unresolved mixture of 10,12-, 9,11-, and 8,10-18:2 as demonstrated previously [14, 17]. A small unknown peak was observed between the *cis,cis* and the *trans,trans* CLA isomer regions. The structural identity of all CLA isomers was established and confirmed by analyzing the DMOX derivatives of selective CLA products by GC-EIMS and GC-DD-FTIR. Representative mass and infrared spectra were published previously [10, 14, 17, 27].

Chromatograms showing the separation of the CLA isomers by Ag^+-HPLC are presented below the GC chromatograms in Figs. 1 to 3. The elution orders of all the geometric (in the order *trans,trans*, *cis/trans*, and *cis,cis*) and positional (in the order 11,13-, 10,12-, 9,11-, and 8,10-18:2) CLA isomers by Ag^+-HPLC were established previously [10]. Ag^+-HPLC was essential to complement the GC analysis and establish the composition of 8 *trans*, 10 *cis*-18:2 and 9 *cis*, 11 *trans*-18:2, and the distribution of most of the *trans,trans* CLA isomers.

In contrast to the commercial CLA preparations, that were found to contain two or four CLA positional isomers, natural dairy products and meats from ruminant animals contain primarily rumenic acid, 9 *cis*, 11 *trans*-18:2 [11, 12, 14, 17–19]. While it has not been established, which isomer(s) is (are) responsible for the reported beneficial properties of CLA, it is generally thought that anticarcinogenicity is due to rumenic acid [12, 15]. The nutritional and physiological effects, if any, of other CLA isomer(s) in commercially available CLA preparations are not known.

We recently reported, that one of the four major *cis/trans* CLA isomers, 11 *cis*, 13 *trans*-18:2, accumulates preferentially in heart phospholipids and specifically in heart and liver diphosphatidylglycerol (DPG) of pigs fed a CLA mixture containing four positional isomers [20]. DPG is a major component of inner mitochondrial membranes and is involved in many enzymes of bioenergetics in the mitochondria [28, 29]. *Watkins* et al. [30] demonstrated that docosahexaenoic acid (22:6 n-3) accumulated in DPG of human colonic adrenocarcinoma (HT-29) cells and increased mito-

chondrial oxidant production. Similarly, 11 *cis*, 13 *trans*-18:2 (or any other CLA isomer incorporated into DPG), could affect mitochondrial oxidant production, particularly since it has been shown that the oxidative susceptibility of CLA is comparable to that of arachidonic acid (20:4 n-6) [31, 32]. In response to our findings that 11 *cis*, 13 *trans*-18:2 was selectively incorporated into DPG [20], a major supplier of commercial CLA preparations recently modified the process to eliminate the 11 *cis*, 13 *trans*-18:2 isomer. The simultaneous elimination of 8 *trans*, 10 *cis*-18:2 from the resulting CLA mixture was an additional benefit of preparing a CLA mixture devoid of 11 *cis*, 13 *trans*-18:2.

In conclusion, the CLA products analyzed in this study were found to contain up to 12 geometric and positional CLA isomers. These findings are based on appropriate and improved analytical methodologies that have only recently been developed [10, 14, 16, 17, 20, 27]. All commercially available CLA products investigated differ, some significantly, and the isomers present may not necessarily represent active CLA components. As new products consisting of two or perhaps only one CLA isomer become available, it will be possible to determine the physiological effects of specific isomers. This is essential for an understanding of this unusual group of lipids.

Abbreviations

Ag^+-HPLC, high-performance liquid chromatography; *cis/trans*, refers to all the CLA isomers having either a *cis,trans* or a *trans,cis* configuration; CLA, conjugated linoleic acid; DMOX, 4,4-dimethyloxazoline; FAME, fatty acid methyl esters; GC-DD-FTIR, gas chromatography-direct deposition-Fourier transform infrared; GC-EIMS, gas chromatography-electron ionization mass spectrometry.

Acknowledgement

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References

[1] *C. Ip, C. Jiang, H.J. Thompson*, and *J.A. Scimeca*, Retention of conjugated linoleic acid in the mammary gland is associated with tumor inhibition during the post-initiation phase of carcinogenesis, Carcinogenesis 18 (1997), 755–759.

[2] *P. Knekt, R. Järvinen, R. Seppänen, E. Pukkala*, and *A. Aromaa*, Intake of dairy products and the risk of breast cancer, Br. J. Cancer 73 (1996), 687–691.

[3] *K.N. Lee, D. Kritchevsky*, and *M.W. Pariza*, Conjugated linoleic acid and atherosclerosis in rabbits, Atherosclerosis 108 (1994), 19–25.

[4] *R.J. Nicolosi, E.J. Rogers, D. Kritchevsky, J.A.Scimeca*, and *P.J. Huth*, Dietary conjugated linoleic acid reduces plasma lipoproteins and early aortic atherosclerosis in hypercholesterolemic hamsters, Artery 22 (1997), 266–277.

[5] *K.L. Houseknecht, J.P. Vanden Heuvel, S.Y. Moya-Camarena, C.P. Portocarrero, L.W. Peck, K.P. Nickel*, and *M.A. Belury*, Dietary conjugated linoleic acid normalizes impaired glucose tolerance in the Zucker diabetic fatty fa/fa rat, Biochem. Biophys. Res. Comm. 244 (1998), 678–682.

[6] *S.F. Chin, J.M. Storkson, K.J. Albright, M.E. Cook*, and *M.W. Pariza*, Conjugated linoeic acid is a growth factor for rats as shown by enhanced weight gain and improved feed efficiency, J. Nutr. 124 (1994), 2344–2349.

[7] *Y. Park, K.J. Albright, W. Liu, J.M. Storkson, M.E. Cook*, and *M.W. Pariza*, Effect of conjugated linoleic acid on body composition in mice, Lipids 32 (1997), 853–858.

[8] *M.E.R. Dugan, J.L. Aalhus, A.L. Schaefer*, and *J.K.G. Kramer*, The effects of conjugated linoleic acid on fat to lean repartitioning and feed conversion in pigs, Can. J. Anim. Sci. 77 (1997), 723–725.

[9] *Y. Li* and *B.A. Watkins*, Conjugated linoleic acids alter bone fatty acid composition and reduce *ex vivo* prostaglandin E_2 biosynthesis in rats fed n-6 or n-3 fatty acids, Lipids 33 (1998), 417–425.

[10] *N. Sehat, M.P. Yurawecz, J.A.G. Roach, M.M. Mossoba, J.K.G. Kramer*, and *Y. Ku*, Silver-ion high-performance liquid chromatographic separation and identification of conjugated linoleic acid isomers, Lipids 33 (1998), 217–221.

[11] *P.W. Parodi*, Conjugated octadecadienoic acids of milk fat, J. Dairy Sci. 60 (1977), 1550–1553.

[12] *S.F. Chin, W. Liu, J.M. Storkson, Y.L. Ha*, and *M.W. Pariza*, Dietary sources of conjugated dienoic isomers of linoleic acid, a newly recognized class of anticarcinogens, J. Food Comp. Anal. 5 (1992), 185–197.

[13] *J.K.G. Kramer, P.W. Parodi, R.G. Jensen, M.M. Mossoba, M.P. Yurawecz*, and *R.O. Adlof*, Rumenic acid: a proposed common name for the major conjugated linoleic acid isomer found in natural products, Lipids 33 (1998), 835.

[14] *M.P. Yurawecz, J.A.G. Roach, N. Sehat, M.M. Mossoba, J.K.G. Kramer, J. Fritsche, H. Steinhart*, and *Y. Ku*, A new conjugated linoleic acid isomer, 7 *trans*, 9 *cis*-octadecadienoic acid, in cow milk, cheese, beef and human milk and adipose tissue, Lipids 33 (1998), 803–809.

[15] *Y.L. Ha, N.K. Grimm*, and *M.W. Pariza*, Newly recognized anticarcinogenic fatty acids: identification and quantification in natural and processed cheeses, J. Agric. Food Chem. 37 (1989), 75–81.

[16] *F. Lavillonnière, J.C. Martin, P. Bougnoux*, and *J.-L. Sébédio*, Analysis of conjugated linoleic acid isomers and content in French cheeses, J. Am. Oil Chem. Soc. 75 (1998), 343–352.

[17] *N. Sehat, J.K.G. Kramer, M.M. Mossoba, M.P. Yurawecz, J.A.G. Roach, K. Eulitz, K.M. Morehouse*, and *Y. Ku*, Identification of conjugated linoleic acid isomers in cheese by gas chromatography, silver ion high performance liquid chromatography and mass spectral reconstructed ion profiles. Comparison of chromatographic elution sequences, Lipids 33 (1988), 963–971.

[18] *A.C. Fogerty, G.L. Ford*, and *D. Svoronos*, Octadeca-9,11-dienoic acid in foodstuffs and in the lipids of human blood and breast milk, Nutr. Rep. Internat. 38 (1988), 937–944.

[19] *H. Lin, T.D. Boylston, M.J. Chang, L.O. Luedecke*, and *T.D. Shultz*, Survey of the conjugated linoleic acid contents of dairy products, J. Dairy Sci. 78 (1995), 2358–2365.

[20] *J.K.G. Kramer, N. Sehat, M.E.R. Dugan, M.M. Mossoba, M.P. Yurawecz, J.A.G. Roach, K. Eulitz, J.L. Aalhus, A.L. Schaefer*, and *Y. Ku*, Distribution of conjugated linoleic acid (CLA) isomers in tissue lipid classes of pigs fed a commercial CLA mixture determined by gas chromatography and silver ion-high performance liquid chromatography, Lipids 33 (1998), 549–558.

[21] *N. Hashimoto, T. Aoyama*, and *T. Shioiri*, New methods and reagents in organic synthesis. 14. A simple efficient preparation of methyl esters with trimethylsilyldiazomethane ($TMSCHN_2$) and its application to gas chromatographic analysis of fatty acids, Chem. Pharm. Bull. 29 (1981), 1475–1478.

[22] *M.P. Yurawecz, J.K. Hood, J.A.G. Roach, M.M. Mossoba, D.H. Daniels, Y. Ku, M.W. Pariza*, and *S.F. Chin*, Conversion of allylic hydroxy oleate to conjugated linoleic acid and methoxy oleate by acid-catalyzed methylation procedures, J. Am. Oil Chem. Soc. 71 (1994), 1149–1155.

[23] *M.M. Mossoba, R.E. McDonald, D.J. Armstrong*, and *S.W. Page*, Identification of minor C_{18} triene and conjugated diene isomers in hydrogenated soybean oil and margarine by GC-MI-FT-IR spectroscopy, J. Chromatogr. Sci. 29 (1991), 324–330.

[24] *M.M. Mossoba*, Applications of capillary GC-FTIR, Inform 4 (1993), 854–859.

[25] *J.K.G. Kramer, V. Fellner, M.E.R. Dugan, F.D. Sauer, M.M. Mossoba*, and *M.P. Yurawecz*, Evaluating acid and base catalysts in the methylation of milk and rumen fatty acids with special emphasis on conjugated dienes and total *trans* fatty acids, Lipids 32 (1997), 1219–1228.

[26] *C.R. Scholfield* and *S. Koritala*, A simple method for preparation of methyl *trans*-10, *cis*-12-octadecadienoate, J. Am. Oil Chem. Soc. 47 (1970), 303.

[27] *N. Sehat, R. Rickert, M.M. Mossoba, J.K.G. Kramer, M.P. Yurawecz, J.A.G. Roach, R.O. Adlof, K.M. Morehouse, J. Fritsche, H. Steinhart,* and *Y. Ku,* Improved separation of conjugated fatty acid methyl esters by silver ion-high performance liquid chromatography, Lipids, in press.
[28] *F.L. Hoch,* Cardiolipins and Biomembrane Function, Biochim. Biophys. Acta 1113 (1992), 71–133.
[29] *M.K. Shigenaga, T.M. Hagen,* and *B.N. Ames,* Oxidative damage and mitochondrial decay in aging, Proc. Natl. Acad. Sci. USA 91 (1994), 10771–10778.
[30] *S.M. Watkins, L.C. Carter,* and *J.B. German,* Docosahexaenoic acid accumulates in cardiolipin and enhances HT-29 cell oxidant production, J. Lipid Res. 39 (1998), 1583–1588.
[31] *J.J.M. Van den Berg, N.E. Cook,* and *D.L. Tribble,* Reinvestigation of the antioxidant properties of conjugated linoleic acid, Lipids 30 (1995), 599–605.
[32] *A. Zhang* and *Z.Y. Chen,* Oxidative stability of conjugated linoleic acids relative to other polyunsaturated fatty acids, J. Am. Oil Chem. Soc. 74 (1997), 1611–1613.

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Chapter 5

Commercial Synthesis of Conjugated Linoleate

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Introduction

Conjugated linoleic acid (CLA) has been available as a health food supplement in soft gelatine capsules since 1995 in the United States, and more recently in several European countries and Japan. CLA products designed for food and animal feed additives are expected to appear on the market in the near future. CLA has been produced for decades for technical purposes and continues to be used as a substitute for Chinese tung oil in the paint and varnish industry due to its "drying" characteristics. The production methods developed for technical CLA products were rapidly modified and improved upon after the discovery of the biological activity of the substance. This chapter will focus on supplements in particular, including current production methods, stability, and breakdown products. Purified isomers are currently available only for research purposes, but a few references to methods available for purification will be given.

CLA for Technical Applications

Dehydration of Ricinoleic Acid

Several decades ago, only two natural oils (tung oil and oiticica) were known to contain conjugated double bonds. Oils that contain these bonds rapidly form a polymer film ("drying") if a thin layer is exposed to air; tung oil was widely used in the paint and varnish industry. An increasing demand for such oils and limited availability encouraged efforts to produce drying oils from nonconjugated oils.

The main constituent of castor bean oil is ricinoleic acid (12-hydroxy-9-octadecenoic acid). Around 1937, dehydrated castor oil appeared on the market in the United States as a substitute for tung oil. Ten years later the product was established as one of the most popular drying oils (1). It has been known since 1888 that castor oil could be dehydrated, and since 1914 it was known that the main isomers of linoleic acid formed had double bonds at positions 9,11 and 9,12, but the detailed composition of dehydrated ricinoleic acid was not investigated until recently. A German patent from 1930 (2) and a U.S. patent from 1934 (3) describe the preparation of dehydrated castor bean oils. A modified procedure was recently used to produce an 83% pure 9-*cis*,11-*trans* CLA concentrate from purified ricinoleic acid (4). Main impurities were the 9-*cis*,11-*cis* and 9-*cis*,12-*trans*-octadecadienoic acids. Conventional dehydration

using high temperatures will create other isomers, mainly 8-*trans*,10-*cis* and *trans*, *trans* isomers. CLA from dehydrated castor oil is not available on the market in supplement form. Apart from safety issues, the reason is the absence of 10-*trans*,12-*cis* CLA, the isomer shown to inhibit fat synthesis (5).

Alkali Isomerization of Linoleic Acid Oils

Attempts to produce drying oil from nonconjugated oils were successful in the late 1930s as well as for oils containing methylene-interrupted fatty acids. In 1941, a U.S. patent was issued that describes the use of monohydric and polyhydric alcohols as solvents and a variety of alkaline catalysts (6). A few years later, two patents were issued that described the use of water (7) and steam (8), respectively, as solvent in an autoclave to achieve the temperatures necessary to conjugate unsaturated acids. It is actually the soap that is conjugated. Upon addition of mineral acid, the conjugated free fatty acids are liberated. Currently, CLA is produced for technical purposes in high alkaline water at ~230°C. Feedstock is usually free fatty acids (after fat splitting to recover glycerol). The product is usually distilled to yield a virtually colorless oil.

Production of CLA for Animal and Human Consumption

Alkaline Water Isomerization

The first products to appear on the health food market contained ~65% CLA, and the profile of the CLA isomers was similar to technical-grade products. Christie *et al.* (9), showed that the main isomers of CLA in addition to 9-*cis*,11-*trans* and 10-*trans*,12-*cis* were an 8,10 and an 11,13 isomer *cis*,*trans* or *trans*,*cis*. These were later identified as 8-*trans*,10-*cis* and 11-*cis*,13-*trans* (10). Such products are still available as supplements, and most if not all are produced from linoleate-rich starting materials in high-alkaline water reactions at temperatures >230°C. We investigated reaction parameters in water alkaline (KOH or NaOH catalyst) reactions trying to avoid formation of 11-*cis*,13-*trans* and 8-*trans*,10-*cis*. It turned out not to be possible to achieve a nearly quantitative isomerization and at the same time avoid formation of the said isomers (data not published).

Isomerization in Propylene Glycol

Quantitative isomerization of oils containing polyunsaturated fatty acids in monohydric and polyhydric alcohols was described in 1941 (6). A detailed procedure using ethylene glycol is described in a patent from 1996 (11). Ethylene glycol has not been used commercially for production of CLA for consumer safety reasons. Propylene glycol has therefore been selected by several producers who independently developed proprietary procedures (12,13). KOH was selected as catalyst because of its high solubility compared with NaOH. Reaction temperatures are typically 130–180°C, and times of reaction are from 3 to >24 h. The quantity of KOH

is substantial and in exce
reaction is complete, th
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to extract CLA and facil
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Thermal [1,5] Sigmatro

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11-*cis*,13-*trans* concentra

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Consumption

contained ~65% CLA, and -grade products. Christie *et* n to 9-*cis*,11-*trans* and 10- *ts* or *trans*,*cis*. These were 0). Such products are still ed from linoleate-rich start- atures >230°C. We investi- KOH catalyst) reactions try- *-cis*. It turned out not to be and at the same time avoid

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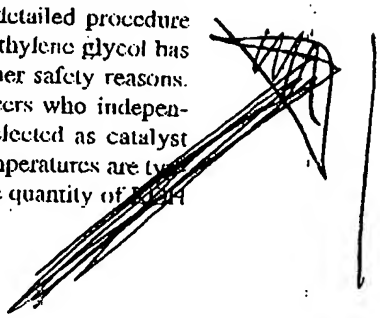

is substantial and in excess of that needed for quantitative saponification. After the reaction is complete, the mixture is cooled down and water and mineral acid (hydrochloric or sulfuric) are added. Free fatty acids of CLA are liberated as soon as the mixture becomes acidic. One patent describes the use of hexane at this point to extract CLA and facilitate separation from the bottom aqueous layer without emulsion problems. However, the operation is possible without the use of hexane. For the sake of recovery of propylene glycol, free fatty acids are preferred as feedstock oil. A triacylglycerol feedstock will create glycerol to contaminate the propylene glycol. After water and solvent (hexane if used) have been removed under vacuum, the CLA product is preferably purified by deodorization and distillation. Peroxides and volatiles are easily removed by deodorization. The peroxides are broken down to secondary volatile products that are removed in the process.

The purification process should also include a molecular distillation step to remove nonvolatile compounds such as polymers, sterols, and propylene glycol esters. Heavy metals could also arise from the isomerization process if mineral acids are used in stainless steel reactors (14). Their concentrations are reduced upon molecular distillation as well. A distilled product is almost colorless and has an acid value of ~200 (mg KOH/g). A nondistilled product might have an acid value of ~190, be yellow to slightly brown in color and have an opaque appearance. However, we have observed a slight decrease in acid value in capsules over time and also a darkening of the oil if the capsule material is colored. Due to the strong alkaline process, free fatty acids are the final product regardless of the form of feedstock (free fatty acid, a monoalkyl ester, or a triacylglycerol oil). Therefore, CLA in supplements are offered almost exclusively as free acids, in contrast to n-3 concentrates that are offered either as ethyl esters or reesterified triacylglycerols.

Isomerization of Mono-Alkyl Esters Using Alkali Metal Alcoholates

Recently, a proprietary method has been developed that quantitatively isomerizes methyl esters and ethyl esters of linoleic acid using very low quantities of catalysts and virtually no solvents (data not published). Because of the quantity of catalyst (~2%), only a small fraction of the ester is saponified and hence appears as free fatty acid after addition of a neutralizing agent. Most of the product (>92%) is still in the form of the methyl or ethyl ester after the isomerization process. The reaction proceeds at temperatures down to below 100°C, and the CLA product is characterized by very low levels of CLA isomers produced by thermal [1,5] sigmatropic rearrangements (see below).

Thermal [1,5] Sigmatropic Rearrangements of CLA Isomers

Production of CLA in propylene glycol or other alcohol under mild conditions gives rise to <0.5% each of the isomers 11-*cis*,13-*trans* and 8-*trans*,10-*cis*. After purification of single isomers, we showed that upon heating to 220°C in an inert atmosphere, 10-*trans*,12-*cis* gives rise to 11-*cis*,13-*trans* (Fig. 5.1). Upon heating an 11-*cis*,13-*trans* concentrate, 10-*trans*,12-*cis* was produced. Under optimal condi-

tions, an equilibrium is established between these isomers, and only minor quantities of *cis,cis* and *trans,trans* isomers are formed. The isomer shift is actually a thermal [1,5] sigmatropic rearrangement, (Fig. 5.2) allowed according to the orbital symmetry theory (Woodward-Hoffmann). For this sigmatropic rearrangement to occur, it is essential that one of the bonds be in the *cis*-configuration. A similar rearrangement is observed for the isomers 9-*cis*,11-*trans* and 8-*trans*,10-*cis* The phenomenon is actually a tool for chemists to produce new isomers. Any given CLA isomer that contains one double bond in the *cis*-configuration and one in the *trans*-configuration can be heated to be isomerized into another specific *cis,trans* or *trans,cis* isomer. Isomers formed might be predicted from formulae as in Fig. 5.2. A simple rule of thumb is that the two double bonds will move against the *cis* end of the bond pairs. For example, 7-*trans*,9-*cis* (a common isomer in milk fat) will isomerize to 8-*cis*,10-*trans* and *vice versa*. Prolonged heating of isomers

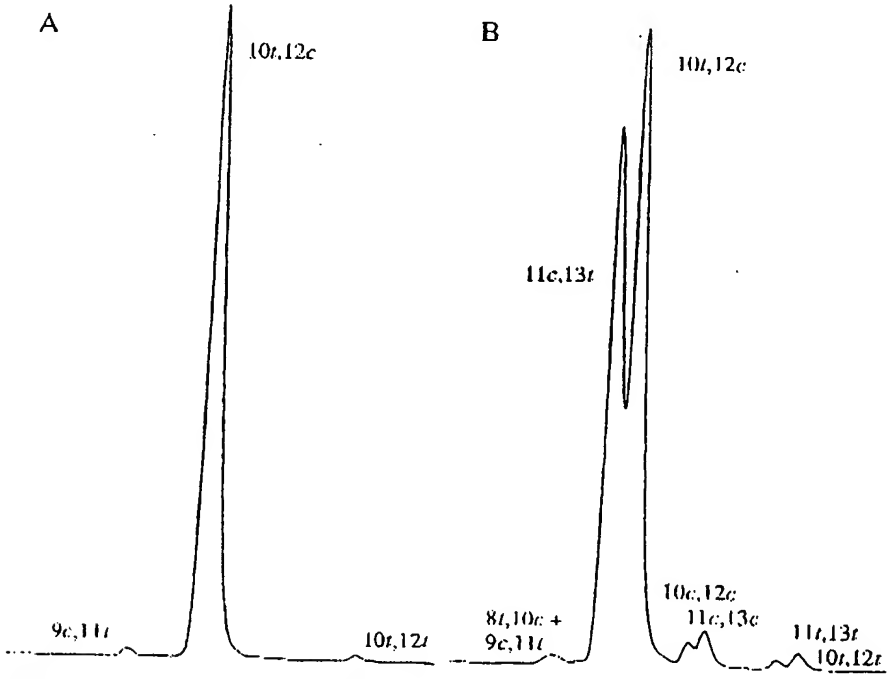

Fig. 5.1. Gas chromatography (GC) profile of ethyl ester of purified 10-*trans*,12-*cis* CLA isomer (a) before and (b) after heating to 220°C in an inert atmosphere for 2 h. The process caused isomerization into the isomer 11-*cis*,13-*trans* by thermal [1,5] sigmatropic hydrogen shift. GC conditions: 100-m CP Sil 88 fused silica capillary column and flame ionization detection (FID).

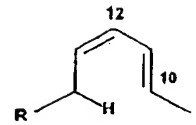

Fig. 5.2. Drawing explain
isomers 10-*trans*,12-*cis* a
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Isomer Profile in Avail

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Stability and Breakd

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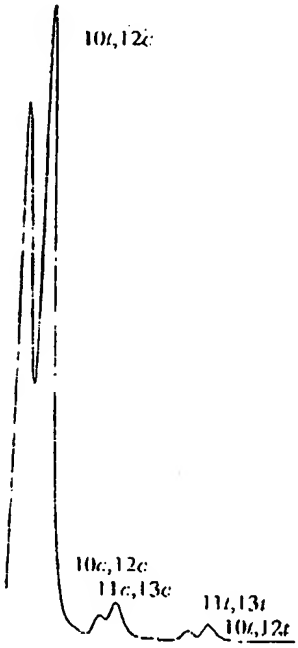

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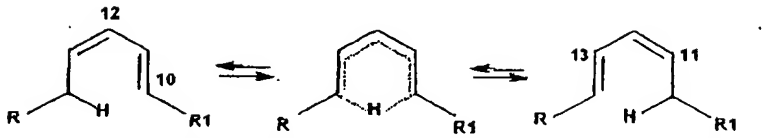

Fig. 5.2. Drawing explaining thermal [1,5] sigmatropic rearrangement between the CLA isomers 10-*trans*,12-*cis* and 11-*cis*,13-*trans*. Reaction is spontaneous and the transition state depicted in the middle is not an intermediate product. R= $-(CH_2)_4$ and R_1 = $-(CH_2)_6(CO_2)H$.

seems to gradually develop *cis*,*cis* and *trans*,*trans* isomers. Impurities present (iron, copper and other metals) will greatly favor formation of *trans*,*trans* isomers.

Isomer Profile in Available Supplements

The total content of CLA in supplements more or less reflects the starting material. Sunflower oil as a starting material results in ~65% CLA, whereas safflower oil yields up to 80%. Both oils contain a level of palmitic acid that tends to cause precipitation below room temperature. Products are now available with a reduced content of saturated acids and >80% CLA. The products can be classified in two groups, the "4-isomer product" and the "2-isomer product" (Fig. 5.3). The latter product contains almost exclusively 9-*cis*,11-*trans* and 10-*trans*,12-*cis*, both up to ~38% of the oil, or almost 50% of the CLA. The former, however, contains several isomers. The elution order on gas chromatography (GC) of the 4 main peaks is 9-*cis*,11-*trans*; 8-*trans*,10-*cis* (may co-elute with 9-*cis*,11-*trans*);11-*cis*,13-*trans*; and 10-*trans*,12-*cis* (9). In addition a major *trans*,*trans* peak (9,11 and 10,12 co-eluting) often reaches the same level. Such products may contain as little as 8% 10-*trans*,12-*cis*. Despite co-elution, the content of 8-*trans*,10-*cis* can be estimated approximately by measurement of 11-*cis*,13-*trans*. Both are produced to the same degree from their mother components. In other words, the ratio of 11-*cis*,13-*trans* to 11-*cis*,13-*trans* + 10-*trans*,12-*cis* equals that of 8-*trans*,10-*cis* to the co-eluting peak 8-*trans*,10-*cis* + 9-*cis*,11-*trans* (data not published). Products from a single source have been reported to show substantial variation in isomer profile (15), and products also are available that contains virtually no (present data, Table 5.1) or totally lack CLA (10). Two of 17 products sampled and analyzed in January-March 2002 by our laboratory contained high levels of the isomers 11-*cis*,13-*trans* and 8-*trans*,10-*cis* (Table 5.1).

Stability and Breakdown Products of CLA Preparations

Stability of CLA Compared with Linoleic Acid

A few studies report data on the stability of CLA compared with linoleic acid in different test models. Bubbling of oxygen through samples at 90°C resulted in a

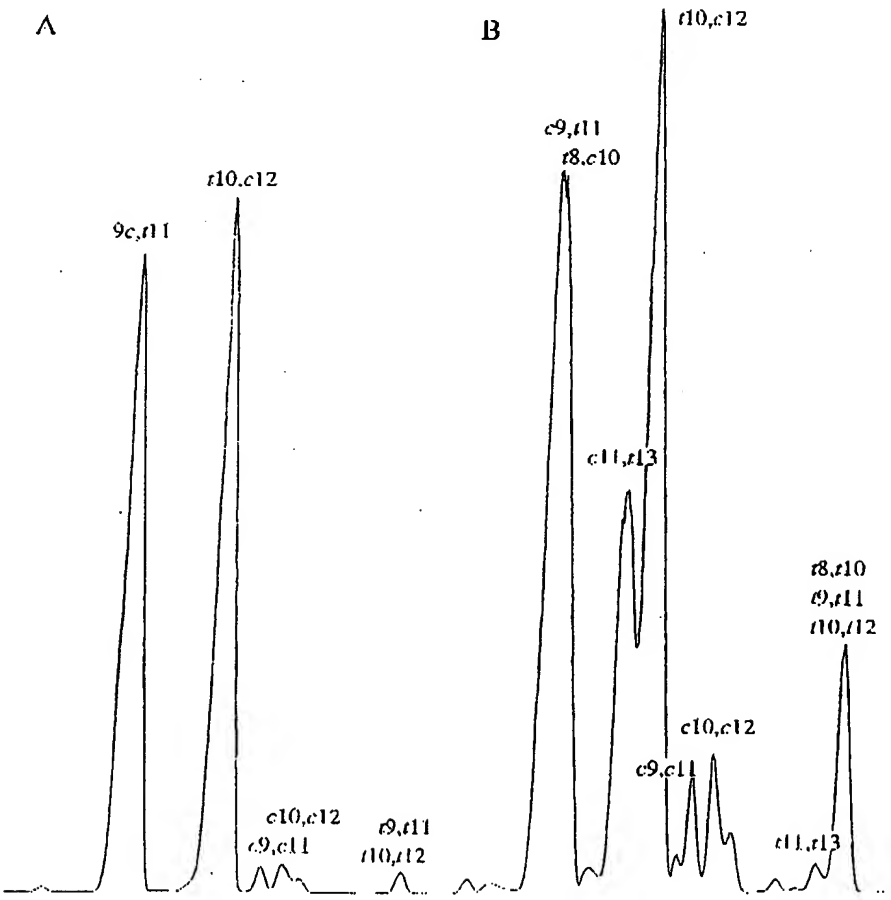

Fig. 5.3. Partial gas chromatography (GC) profile of ethyl esters of (a) a "2 isomer type" and (b) a "4 isomer type" CLA supplement, using a 100-m CP Sil 88 fused silica capillary column and flame ionization detection (FID). Product (a) is identical to product No. 14 and product (b) is identical to No.17 in Table 5.1. Note co-elution of 8-*trans*,10-*cis* and 9-*cis*,11-*trans*.

much higher peroxide value (PV) in linoleic acid (16) than for CLA. When a mixture of CLA isomers was heated to 50°C in air, the rate of oxidation was considerably faster for CLA than for linoleic acid. The rate of oxidation was measured as "remaining CLA" by GC. When comparing groups of CLA isomers, stability decreased in order of *trans,trans* > *cis,trans* or *trans,cis* > *cis,cis*. (17). In a study in aqueous and solvent systems measuring stability by the induction period system,

TABLE 5.1
Content of CLA (% of Tot.
January–March 2002[a]

Product	Product type
1	Soft gelatine cap
2	Liquid
3	Soft gelatine cap
4	Soft gelatine cap
5	Soft gelatine cap
6	Soft gelatine cap
7	Soft gelatine cap
8	Soft gelatine cap
9	Soft gelatine cap
10	Soft gelatine cap
11	Soft gelatine cap
12	Soft gelatine cap
13	Soft gelatine cap
14	Soft gelatine cap
15	Liquid, emulsion
16	Soft gelatine cap
17	Soft gelatine cap

[a]The isomers 10-*trans*,12-*cis* and
ucts were of the "4 isomer" type.
9-*cis*,11-*trans* (not tabulated due
trans,12-*cis* in all supplements co
mg KOH/g. (A 100.00% free fatty
region of product 14 and product

CLA was more stable tha
esters (18). Another stud
the following order: oleat
40°C and monitored by
cis,11-*trans*, the major m
and 13-monohydroperox
13-, and 14-monohydrope

Data reported on the
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seems comparable to that
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Volatiles

In a pilot project on deve
hexane was observed in
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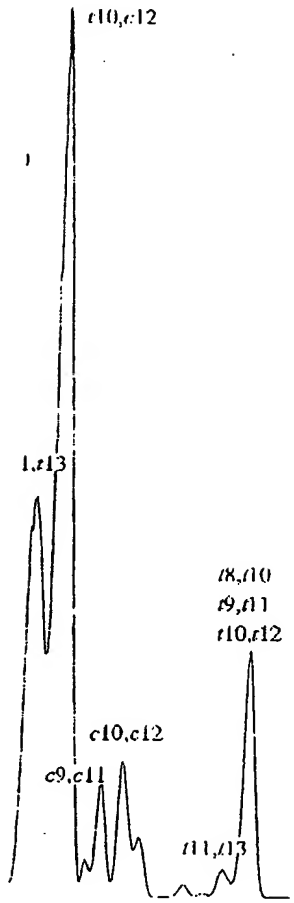

yl esters of (a) a "2 isomer 100-m CP Sil 88 fused silica duct (a) is identical to prod- 5.1. Note co-elution of 8-

ıan for CLA. When a mix- of oxidation was consider oxidation was measured as of CLA isomers, stability s > *cis,cis*. (17). In a study ıe induction period system,

TABLE 5.1
Content of CLA (% of Total) in 17 Commercial Supplements Sampled in January–March 2002[a]

Product	Product type	Country	%CLA	%10*t*,12*c*	%11*c*,13*t*	Acid value
1	Soft gelatine capsule	Norway	80.1	47.8	0.4	197
2	Liquid	Norway	78.6	47.1	1.8	?
3	Soft gelatine capsule	Norway	69.1	46.7	1.2	196
4	Soft gelatine capsule	Norway	78.3	48.7	0.3	197
5	Soft gelatine capsule	Norway	76.4	46.6	1.3	193
6	Soft gelatine capsule	U.S.	71.4	46.3	0.5	189
7	Soft gelatine capsule	U.S.	74.8	43.1	0.9	192
8	Soft gelatine capsule	U.S.	77.9	48.5	0.3	199
9	Soft gelatine capsule	U.S.	70.8	44.4	0.6	189
10	Soft gelatine capsule	U.S.	79.6	45.3	0.4	193
11	Soft gelatine capsule	U.S.	77.0	44.4	2.3	192
12	Soft gelatine capsule	U.S.	74.3	43.6	1.0	187
13	Soft gelatine capsule	U.S.	61.5	28.5	0.8	180
14	Soft gelatine capsule	U.S.	76.3	48.4	0.3	196
15	Liquid, emulsion	U.S.	1.2	47.8	0.3	NA
16	Soft gelatine capsule	S. Africa	51.7	16.5	16.1	198
17	Soft gelatine capsule	Norway	57.7	29.9	16.5	200

[a]The isomers 10-*trans*,12-*cis* and 11-*cis*,13-*trans* are expressed as the percentage of total CLA. Only two products were of the "4 isomer" type. Two products were liquids, one oil and one emulsion (1.7% fat). Content of 9-*cis*,11-*trans* (not tabulated due to overlap with 8-*trans*,10-*cis*) is approximately equal or slightly less than 10-*trans*,12-*cis* in all supplements currently available. Distilled products typically have acid values of 195–200 mg KOH/g. (A 100.00% free fatty acid product of oleic acid has a theoretical acid value of 198.60). GLA region of product 14 and product 17 is illustrated in Figure 5.3. NA, not available.

CLA was more stable than linoleic acid as free fatty acids, and less stable as ethyl esters (18). Another study using methyl esters reported that stability decreased in the following order: oleate > CLA > linoleate. Samples were stored in the dark at 40°C and monitored by thin-layer chromatography (TLC), GC and PV. From 9-*cis*,11-*trans*, the major monohydroperoxides formed were identified as 8-, 9-, 12- and 13-monohydroperoxides, whereas 10-*trans*,12-*cis* yielded primarily 9-, 10-, 13-, and 14-monohydroperoxides (19).

Data reported on the PV of CLA preparations are consistent with our observations. CLA do not easily develop high PV, yet the oxidative breakdown of CLA seems comparable to that of linoleic acid. The reason is likely to be a more rapid breakdown of peroxides into secondary oxidation products.

Volatiles

In a pilot project on developing a procedure for CLA production, a high content of hexane was observed in a product by headspace GC-mass spectrometry. After searching for the source of contamination, it was finally concluded that pentane

and hexane are among the secondary oxidation products of CLA. This was later confirmed by experiments. To our knowledge, hexane has never been reported to be an important inherent oxidation product of vegetable oils. In a free fatty acid concentrate of 9-*cis*,11-*trans* stored in the dark with air access for 1 wk, the two major volatiles that developed were, not surprisingly, heptanal and 2-nonenal. The concentration increased from 4.8 and 0.7 to 84.6 and 22.5 µg/g, respectively. Volatile breakdown products seem not to build up in soft gelatine capsule supplements. A CLA product that was stored for 5 y at room temperature contained 2.3 µg/g hexanal and 2.2 µg/g heptanal (data not published). No antioxidant was added to the supplement.

Among less volatile breakdown products, furan fatty acids were reported when air was bubbled through CLA dissolved in a mixture of methanol and water at 50°C. (20). Furanoid fatty acids might also arise in preparation of fatty acid methyl esters (FAME) for GC. To our knowledge, furan fatty acids have not been reported as an oxidative breakdown product in dry oil preparations of CLA.

Polymers

Conjugated oils are considered valuable raw materials for the paint and varnish industry because of their film forming properties ("drying") upon air access. This property gives rise to concern regarding the stability of CLA preparations. In a stability test program, 10 mL of CLA triacylglycerols and free fatty acids were stored in an amber open glass bottle in darkness. After 4 mo at 25°C, controls without antioxidants added were highly viscous and not suitable for further stability testing. The samples had a membrane layer on the surface, and the viscosity clearly developed over time. Samples with antioxidants did show a retarded viscosity development (data not published).

Soft gelatine capsules are considered to give reasonable protection from exposure of unsaturated oils to air. Capsules containing CLA free fatty acids showed a slight increase in polymer content from 1% in freshly prepared capsules to 7% after 5 y (data not published). For comparison of health risks, a limit for rejection on cooking oils has been established in some countries; values listed in a report from the European Parliament are 16% (Holland), and 10% (Belgium and Czech Republic) (21).

Stability of CLA in Soft Gelatine Capsules

No data have yet been published on the stability of CLA in capsules. Observations on polymers and volatiles in capsules are reported above. In a stability test program according to International Conference on Harmonization (ICH) guidelines on a free fatty acid product, the content of total CLA was not significantly reduced after 24 mo at 25°C/60% relative humidity. In this test, CLA was measured by GC. Peroxide value (PV) did not develop in the capsules (data not published).

Next Generation Pr

Isomer Purification

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Summary

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Next Generation Products

Isomer Purification

All CLA supplements currently offered contain approximately equal amounts of 9-*cis*,11-*trans* and 10-*trans*,12-*cis*. The extra costs of producing a biased isomer product might be justified if beneficial health effects were documented. The 9-*cis*,11-*trans* and the 10-*trans*,12-*cis* isomers of CLA are now available for research purposes in kilogram scale with a purity of ~90%. In small quantities, purities up to 99% are offered. High yields and high purity can be obtained by repeated crystallization of the methyl ester forms in acetone at temperatures as low as –60°C (22).

A concentrate with 83% 9-*cis*,11-*trans* isomer was obtained from gentle dehydration of ricinoleic acid from castor bean oil and subsequent purification steps (4). The use of urea inclusion compounds does not seem to be a feasible procedure to separate 9-*cis*,11-*trans* and 10-*trans*,12-*cis* (23). Enzymes, however, are promising tools for these separations. A 98% concentrate of 9-*cis*,11-*trans* was reported by using lipase from *Geotrichum candidum*. The enzyme was capable of esterifying selectively 9-*cis*,11-*trans* to monohydric alcohols from a mixture of several isomers (24). A patent has been issued on purification and characterization of isomerases from *Propionibacterium acnes* and *Clostridium sporogenes*. The purified isomerase preparations were able to quantitatively isomerize linoleic acid into the 10-*trans*,12-*cis* isomer of CLA (25).

Triacylglycerols for Food Applications

Free fatty acids and monoalkyl esters are applicable to supplement capsules and probably also to animal feed formulations. However, as an ingredient in food for human consumption, CLA is most attractive as a triacylglycerol. A nonspecific lipase has been reported to esterify CLA with glycerol very efficiently (26). Incorporation of CLA into food fats and oils has also been reported for fish oils (27), butterfat (28,29), and corn oil (30). A bottled triacylglycerol product, stabilized with antioxidants, has been available in the health food market in Scandinavia since 2000. Flavor and antioxidants are added to the oil designed to be taken by spoon. Further technical developments of CLA products improving the stability and applicability as well as addressing specific issues of food legislation will require attention before CLA can be made available as an ingredient for animal feed and human food.

Summary

CLA supplements for human consumption have been available since 1995, and most of the products contain between 60 and 80% CLA in the form of free fatty acids. The history of CLA produced for technical purposes dates back almost 100

y, however. The isomer profile of the supplements range from an almost pure 9-*cis*,11-*trans* + 10-*trans*,12-*cis*-50/50 mixture (made in alcohol solvents between 100 and 150°C), to a mixture with four prominent *cis,trans* or *trans,cis* isomers produced in high alkaline water at high temperatures, of which 8-*trans*,10-*cis* and 11-*cis*,13-*trans* 18:2 are produced from 9-*cis*,11-*trans* and 10-*trans*,12-*cis*, respectively, by thermal [1,5] sigmatropic rearrangements of the isomers. Supplements are typically offered as free fatty acids in soft gelatine capsules. Unpublished data on stability of CLA in capsules stored according to ICH guidelines for 2 y did not show any loss of active ingredient.

Acknowledgments

Per Christian Sæbø and his staff at the laboratory of Natural ASA is acknowledged for patient experimental work on CLA production and purification process developments for the last 5 years. Thanks to Prof. emeritus Lars Skattebøl for valuable comments on migration of sigma bonds.

References

1. Radlove, S.B., DeJong, V.M., and Falkenburg, L.B. (1948) A Continuous Process for the Dehydration of Castor Oil, *J. Am. Oil Chem. Soc. 25*, 267–271.
2. Scheiber, J., Patentschrift, No. 513540 (1930).
3. Scheiber, J., U.S. Patent 1,942,778 (1934).
4. Berdeaux, O., Christie, W.W., Gunstone, F.D., and Sébédio, J.-L. (1997) Large-Scale Synthesis of Methyl *cis*-9, *trans*-11-Octadecadienoate from Methyl Ricinoleate, *J. Am. Oil Chem. Soc. 74*, 1011–1015.
5. Pariza, M.W., Park, Y., and Cook, M.E. (2001) The Biologically Active Isomers of Conjugated Linoleic Acid, *Prog. Lipid Res. 40*, 283–298.
6. Burr, O.G., U.S. Patent 2,242,230 (1941).
7. Bradley, T.F., U.S. Patent 2,350,583 (1944).
8. Kirschenbauer, H.G., Allendale, N.J., U.S. Patent 2,3893260 (1945).
9. Christie, W.W., Dobson, G., and Gunstone, F.D. (1997) Isomers in Commercial Samples of Conjugated Linoleic Acid, *Lipids 32*, 1231.
10. Yurawecz, M.P., Sehat, N., Mossoba, M.M., Roach, J.A.G., Kramer, J.K.G., and Ku, Y. (1999) Variations in Isomers Distribution in Commercially Available Conjugated Linoleic Acid, *Fett/Lipid 101*, 277–282.
11. Cook, M.E., Pariza, M.W., Lee, K.N., Wentworth, B.C., U.S. Patent 5,504,114 (1996).
12. Iwata, T., Kamegai, T., Sato, Y., Watanabe, K., and Kasai, M., U.S. Patent 5,986,116 (1999).
13. Bhuggan, K., Cain, F.W., Harris, J.B., and Taran, V., European Patent 0 902 082 A1 (1999).
14. Reaney, M.J.T., Liu, Y.-D., and Westcott, N.D. (1999) Commercial Production of Conjugated Linoleic Acid, in *Advances in Conjugated Linoleic Acid Research*, Vol. 1 (Yurawecz, M.P., Mossoba, M.M., Kramer, J.K.G., Pariza, M.W., and Nelson, G.N., eds.) pp. 39–54, AOCS Press, Champaign, IL.
15. Adlof, R.O., Copes, L.C., and Walter, E.L. (2001) Changes in Conjugated Linoleic Acid Composition Within Samples Obtained from a Single Source, *Lipids 36*, 315–317.
16. Allen, R.R., Jackson, A of Highly Unsaturated Nonconjugated Linoleic
17. Yang, L., Leung, L.K Conjugated Linoleic A
18. Seo, H.-S., Endo, Y., Conjugated Linoleic A
19. Hämäläinen, T.I., Sund Hydroperoxide Format Ester, *Eur. J. Lipid Sci.*
20. Yurawecz, M.P., Hood Fatty Acids Determine *Lipids 30*, 595–598.
21. Boatella Riera, J., Code Oils: Assessment of R Parliament, pp. 3–96, Di
22. Berdeaux, O., Voinot, Preparation of Methyl Methyl Linoleate, *J. An*
23. Strocchi, A., and Bona and Conformational S *Phys. Lipids 15*, 87–94.
24. Haas, M.J., Kramer, J.F Mossoba, M.M., and Conjugated Linoleic A
25. Rosson, R.A., Deng, X Patent 01/00846 A2 (20
26. Arcos, J.A., Otero, C Acylglycerols from Co *Biotechnol. Lett. 20*, 61
27. Garcia, H.S., Arcos, J Containing n-3 Fatty A of Fish Oil, *Biotechnol.*
28. Garcia, H.S., Keough (Acidolysis) of Butterf *Sci. 83*, 371–377.
29. Garcia, H.S., Storkso Butteroil with Conjuga Reactions, *Biotechnol.*
30. Martinez, C.E., Vinay, Catalyzed Interesterific Organic Solvents, *Food*

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U.S. Patent 5,504,114 (1996)
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) Commercial Production of
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za, M.W., and Nelson, G.N.,

nges in Conjugated Linoleic
e Source, *Lipids 36*, 315–317.

16. Allen, R.R., Jackson, A., and Kuromerow, F.A. (1949) Factors Which Affect the Stability of Highly Unsaturated Fatty Acids. I. Differences in the Oxidation of Conjugated and Nonconjugated Linoleic Acid, *J. Am. Oil Chem. Soc. 26*, 395–399.
17. Yang, L., Leung, L.K., Huang, Y., and Chen, Z-Y. (2000) Oxidative Stability of Conjugated Linoleic Acid Isomers, *J. Agric. Food Chem. 48*, 3072–3076.
18. Seo, H.-S., Endo, Y., and Fujimoto, K. (1999) Kinetics for the Autoxidation of Conjugated Linoleic Acid, *Biosci. Biotechnol. Biochem. 63*, 2009–2010.
19. Hämäläinen, T.L, Sundberg, S., Mäkinen, M., Kaltia, S., Hase, T., and Hopia, A. (2001) Hydroperoxide Formation During Autoxidation of Conjugated Linoleic Acid Methyl Ester, *Eur. J. Lipid Sci. Technol. 103*, 588–593.
20. Yurawecz, M.P., Hood, J.K., Mossoba, M.M., Roach, J.A.G., and Ku, Y. (1995) Furan Fatty Acids Determined as Oxidation Products of Conjugated Octadecadienoic Acid, *Lipids 30*, 595–598.
21. Boatella Riera, J., Codony, R., Rafecas, M., and Guardiola, F. (2000) Recycled Cooking Oils: Assessment of Risks for Public Health, Document Published by the European Parliament, pp. 3–96, Directorate General for Research, Directorate A, Luxembourg.
22. Berdeaux, O., Voinot, L., Juanèda, P., and Sébédio, J.-L. (1998) A Simple Method of Preparation of Methyl *trans*-10,*cis*-12 and *cis*-9, *trans*-11-Octadecadienoates from Methyl Linoleate, *J. Am. Oil Chem. Soc. 75*, 1749–1755.
23. Strocchi, A., and Bonaga, G. (1975) Correlation Between Urea Inclusion Compounds and Conformational Structure of Unsaturated C_{18} Fatty Acid Methyl Esters, *Chem. Phys. Lipids 15*, 87–94.
24. Haas, M.J., Kramer, J.K.G., McNeill, G., Scott, K., Foglia, T.A., Sehat, N., Fritsche, K., Mossoba, M.M., and Yurawecz, M.P. (1999) Lipase-Catalyzed Fractionation of Conjugated Linoleic Acid Isomers, *Lipids 34*, 979–987.
25. Rosson, R.A., Deng, M.-D., Grund, A.D., and Peng, S.S., Linoleate Isomerase, WO Patent 01/00846 A2 (2001).
26. Arcos, J.A., Otero, C., and Hill, C.G. (1998) Rapid Enzymatic Production of Acylglycerols from Conjugated Linoleic Acid and Glycerol in a Solvent-Free System, *Biotechnol. Lett. 20*, 617–621.
27. Garcia, H.S., Arcos, J.A., Ward, D.J., and Hill, C.G. (2000) Synthesis of Glycerides Containing n-3 Fatty Acids and Conjugated Linoleic Acid by Solvent-Free Acidolysis of Fish Oil, *Biotechnol. Bioeng. 70*, 587–591.
28. Garcia, H.S., Keough, K.J., Arcos, J.A., and Hill, C.G. (2000) Interesterification (Acidolysis) of Butterfat with Conjugated Linoleic Acid in a Batch Reactor, *J. Dairy Sci. 83*, 371–377.
29. Garcia, H.S., Storkson, J.M., Pariza, M.W., and Hill, C.G. (1998) Enrichment of Butteroil with Conjugated Linoleic Acid Via Enzymatic Interesterification (Acidolysis) Reactions, *Biotechnol. Lett. 20*, 393–395.
30. Martinez, C.E., Vinay, J.C., Brieva, R., Hill, C.G., and Garcia, H.S. (1999) Lipase-Catalyzed Interesterification (Acidolysis) of Corn Oil and Conjugated Linoleic Acid in Organic Solvents, *Food Biotechnol. 13*, 183–193.

Conjugated Linoleic Acid Modulates Tissue Levels of Chemical Mediators and Immunoglobulins in Rats

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ABSTRACT: The effects of conjugated linoleic acid (CLA) on the levels of chemical mediators in peritoneal exudate cells, spleen and lung, and the concentration of immunoglobulins in mesenteric lymph node and splenic lymphocytes and in serum were examined in rats. After feeding diets containing either 0 (control), 0.5 or 1.0% CLA for 3 wk, there was a trend toward a reduction in the release of leukotriene B_4 (LTB_4) from the exudate cells in response to the dietary CLA levels. However, CLA did not appear to affect the release of histamine. A similar dose-response pattern also was observed in splenic LTB_4, lung LTC_4 and serum prostaglandin E_2 levels, and the differences in these indices between the control and 1.0% CLA groups were all statistically significant. The reduction by CLA of the proportions of n-6 polyunsaturated fatty acids in peritoneal exudate cells and splenic lymphocyte total lipids seems to be responsible at least in part for the reduced eicosanoid levels. Splenic levels of immunoglobulin A (IgA), IgG, and IgM increased while those of IgE decreased significantly in animals fed the 1.0% CLA diet. This was reflected in the serum levels of immunoglobulins. The levels of IgA, IgG, and IgM in mesenteric lymph node lymphocytes increased in a dose-dependent manner, while IgE was reduced in those fed the higher CLA intake. However, no differences were seen in the proportion of T-lymphocyte subsets of mesenteric lymph node. These results support the view that CLA mitigates the food-induced allergic reaction.
Lipids 33, 521–527 (1998).

Conjugated linoleic acid (conjugated derivatives of linoleic acid, CLA) exerts diverse physiological effects most of which are favorable to human health. A range of studies has shown a marked alleviating effect of CLA on mammary carcinogenesis (1–4). The mechanism underlying this effect is not yet well understood (5), but continued intake of CLA is not necessarily required for suppression of carcinogenesis (6,7). When considering the diverse effects of CLA, it is reasonable that eicosanoids are involved in the mechanism. The influence of CLA on the metabolic processes leading from linoleic acid to arachidonic acid and, hence, eicosanoids appears to be related to their desirable effects, since CLA tended to reduce the tissue level of prostaglandin E_2 (PGE_2), a putative candidate for a cancer-promoting effect of dietary n-6 polyunsaturated fatty acids (PUFA) (8). In addition, there is a possibility that CLA itself serves as substrate of enzymatic systems for eicosanoid production, as it is shown to undergo desaturation and elongation similar to linoleic acid (9), although it is unknown whether these metabolites could be converted to eicosanoids.

Since the food allergic reaction can readily be modified by the type of dietary PUFA, either n-6 or n-3 (10,11), it is interesting to know if CLA could modify it. The clinical symptom of food allergy is induced by the production of chemical mediators such as histamine and leukotriene (LT) and PG triggered by allergen-specific immunoglobulin (Ig)E (12,13). Our previous studies showed a reduction by CLA of the serum PGE_2 level (8), which is one of the typical chemical mediators in the allergic reaction (12,13). In this context, Belury and Kempa-Steczko (14) showed that CLA reduces the proportion of linoleic acid dose-dependently in hepatic phospholipid and suggested this may result in modified arachidonate-derived eicosanoid production by extrahepatic tissues. More recently, Wong *et al.* (15) reported that CLA modulates certain aspects of the immune defense such as lymphocyte proliferation in mice, although the effect was not always reproduced possibly because of the dependence on the duration of the feeding period. In the present study, we measured the production of chemical mediators and the level of Ig in rats fed different levels of CLA, either 0.5 or 1.0%.

MATERIALS AND METHODS

Preparation of CLA. CLA was prepared according to the method described by Ip *et al.* (16). In brief, 50 g of linoleic acid, purity >99% (Sigma Chemical Co., St. Louis, MO) was dissolved in 290 g of ethylene glycol containing 15 g of NaOH and heated at 180°C for 2 h under nitrogen. After cooling to room temperature, the content was adjusted to pH 4 and extracted with *n*-hexane. The hexane layer was washed with 5% NaCl, dehydrated with 3-A molecular sieves (Nacalai

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Abbreviations: CLA, conjugated linoleic acid; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; LT, leukotriene; MLN, mesenteric lymph node; PEC, peritoneal exudate cells; PG, prostaglandin; POD, peroxidase; PUFA, polyunsaturated fatty acid.

Tesqu, Kyoto, Japan) and dried in a rotary evaporator under nitrogen. The purity of CLA was measured by gas–liquid chromatography (Shimadzu GC-17A, Kyoto, Japan) using a Supelcowax 10 column (0.32 mm × 60 m, film thickness, 0.25 μm; Supelco Inc., Bellefonte, PA). Column temperature was raised from 150 to 220°C at a rate of 4°C/min. The identification of peaks was carried out by the equivalent chain length method (17) and gas chromatography–mass spectrometry (Jeol Auto MS 50, Tokyo, Japan). The purity of CLA preparation was 80.7% with the following composition in percentage: 9*c*,11*t*/9*t*,11*c*, 29.8; 10*t*,12*c*, 29.6; 9*c*,11*c*, 1.3; 10*c*,12*c*, 1.4; 9*t*,11*t*/10*t*,12*t*, 18.6; linoleic acid, 5.6; and others, 13.7.

Animals and diets. The animal experiment adhered to the Kyushu University guidelines for the care and use of laboratory animals. Male, 4-wk-old Sprague-Dawley rats were obtained from Japan Charles River (Atsugi, Japan) and housed individually in a room with controlled temperature and light (20–23°C and lights on 0800–2000 h). After acclimation for 4 d, rats were divided into three groups of 10 rats which were given free access to the experimental diets. The diets were prepared according to the recommendation of the American Institute of Nutrition (AIN-93G diet) (18). The basal diet contained the following ingredients, in g/100 g diet: cornstarch 39.8; casein, 20.0; dextrinized cornstarch, 13.2; sucrose, 10.0; soybean oil, 7.0; AIN-93 mineral mixture, 3.5; AIN-93 vitamin mixture, 1.0; L-cystine, 0.3; choline bitartrate, 0.25; cellulose, 5.0; *tert*-butylhydroquinone, 0.002; and either linoleic acid, 1.0; linoleic acid (Control) and CLA, 0.5 and 0.5; or CLA, 1.0. Thus, LA or CLA was added at the expense of soybean oil in the AIN-93G diet. The fatty acid composition calculated from the composition of individual oils is given in Table 1. Body weight and food intake were recorded every other day. After 3 wk of feeding, five rats were used for collection of the exudate cells and the remaining five rats for other analyses. Blood was withdrawn from the abdominal aorta under light diethyl ether anesthesia and tissues were immediately excised.

Preparation of peritoneal exudate cells (PEC). The method described by Matsuo *et al.* (19) was adopted for the preparation of PEC. Tyrode buffer, consisting of 137 mM NaCl, 2.7 mM KCl, 0.4 mM $NaH_2PO_4 \cdot 2H_2O$, 1 mM $MgCl_2 \cdot 6H_2O$, 12 mM $NaHCO_3$, 1.8 mM $CaCl_2 \cdot 2H_2O$, 5.6 mM D-glucose and 0.1% bovine serum fraction V (Boehringer Mannheim GmbH, Mannheim, Germany), pH 7.4, was injected into the rat peritoneal cavity (6 mL/100 g body weight), and the abdomen was gently massaged for 2 min. Then, the cavity was opened, and the buffer containing PEC was recovered with a plastic pipet. The fluid was centrifuged at 200 × *g* for 5 min at 4°C. The supernatant was discarded and the cell pellet was resuspended in Tyrode buffer.

TABLE 1
Fatty Acid Composition of Dietary Fat[a]

Fatty acid	Group		
	Control	0.5% CLA	1.0% CLA
16:0	9.1	9.0	9.0
18:0	3.2	3.42	3.2
18:1	20.4	20.3	20.1
CLA	—	6.4	12.9
18:2	59.7	53.6	47.4
18:3	7.5	7.5	7.4

[a]Fatty acid composition was calculated from the composition of individual component fats, soybean oil, linoleic acid, and conjugated linoleic acid (CLA).

Measurement of leukotriene B_4 (LTB_4) and histamine. LTB_4 was measured as described elsewhere (20–22). PEC (2×10^6 cells) were suspended in Tyrode buffer containing 5 mM calcium ionophore A23187 (Sigma Chemical Co.). After incubating for 20 min at 37°C, 50 mL of the acetonitrile/methanol mixture (6:5, vol/vol) and 50 ng of PGB_2 (Sigma Chemical Co.), as the internal standard, were added. The mixture was kept at –20°C for 30 min and then centrifuged at 1,000 × *g* for 10 min. The supernatant was filtered through a 4-GV 0.22 μm filter (Millipore Corp., Tokyo, Japan). LTB_4 was measured by reversed-phase high-performance liquid chromatography (HPLC) (SCL-10A; Shimadzu Co., Kyoto, Japan) equipped with an ODS-A column (150 × 6.0 mm, 5 μL particle size; YMC, Kyoto, Japan). A mixture of acetonitrile/methanol/water (30:25:45, by vol) containing 5 mM CH_3COONH_4 and 1 mM disodium EDTA, pH 5.6, was used as a mobile phase with a flow rate of 1.1 mL/min. LTB_4 and PGB_2 were detected by absorbance at 280 nm (SPD-10A; Shimadzu Co.). Quantitation of LTB_4 was achieved by comparing the peak area of LTB_4 with that of PGB_2. Histamine content in the culture supernatant was measured fluorometrically (19,23). The intracellular content of histamine also was measured after disrupting the cells by sonication.

Preparation of spleen and mesenteric lymph node (MLN) lymphocytes. Spleen and MLN were excised immediately after withdrawing blood from the aorta, and the tissues were immersed in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) (24,25). The lymphocytes were rinsed three times with the RPMI 1640 medium and filtered to remove tissue scum. To remove fibroblasts, cell suspension was incubated for 30 min at 37°C. Then, 5 mL of the resulting cell suspension was layered on 4 mL of Lympholyte-Rat (Cedarlane, Hornby, Canada) and centrifuged at 1,500 × *g* for 30 min. The lymphocyte band at the interface was recovered, and the cells were rinsed again. The lymphocytes were cultured in 10% fetal bovine serum (Intergen, Purchase, NY) in RPMI 1640 medium at a cell density of 2.5×10^6 cells/mL with or without 2.5 μg/mL of lipopolysaccharide (Bacto lipopolysaccharide B, *Escherichia coli* 026:B6; Difco Laboratories, Detroit, MI). After incubation at 37°C for 24 and 72 h, the concentrations of IgA, IgG, IgM, and IgE were measured by an enzyme-linked immunosorbent assay (ELISA) (26).

T-cell population analysis. Spleen and MLN lymphocytes were analyzed as $CD4^+$- and $CD8^+$-cells by using fluorescein-labeled mouse anti-CD4 (W3/25, mouse IgG1) or phycoerythrin-labeled mouse anti-CD8 (MRC OX-8, mouse IgG1) (both from Serotec Ltd., Kidlington, Oxford, United King-

dom) (23,25). The stained lymphocytes were fixed with 2% paraformaldehyde and analyzed with the EPICS Profile II flowcytometer (Coulter Electronics Ltd., Luto, United Kingdom).

Measurement of serum and culture supernatant Ig by ELISA. Measurements of total Ig were executed using sandwich ELISA methods (24,25). Goat anti-rat IgE, rabbit anti-rat IgG (Fab')2, goat anti-rat IgM (all from Biosoft, Paris, France), and mouse anti-rat IgA (Zymed Lab, San Francisco, CA) were used to fix respective Ig. These antibodies were diluted 1,000 times with 50 mM carbonate-bicarbonate buffer (pH 9.6), and each well of 96-well plates was treated with 100 µL of the solution for 1 h (2 h for IgA) at 37°C. After blocking with 300 µL of the blocking solution overnight at 4°C, each well was treated with 100 µL of the diluted serum or culture supernatant for 1 h (2 h for IgA) at 37°C. Bound IgA was detected by reacting stepwise with 100 µL of peroxidase (POD)-conjugated rabbit anti-rat IgA (1,000 times dilution; Zymed) at 37°C for 2 h, IgG with 100 µL of POD-conjugated rabbit anti-rat IgG (Fab')2 (2,000 times dilution; Cappel, West Chester, PA), and IgM with 100 µL of POD-conjugated goat anti-rat IgM (1,000 times dilution, Cappel) at 37°C for 1 h. Wells were rinsed three times with Tween-20 in phosphate-buffered saline between each step. After incubation at 37°C for 15 min with 100 µL of 1.5% oxalic acid, absorbance at 415 nm was measured with an MPR-A4i ELISA reader (Tosoh, Tokyo, Japan). The bound IgE was detected by reacting with biotin-conjugated mouse anti-rat IgE (2,000 times dilution; Betyl, Montgomery, TX) followed by POD-conjugated avidin (5,000 times dilution, Zymed Lab) at 37°C for 1 h.

Statistical analysis. Data were analyzed by one-way analysis of variance followed by Duncan's new multiple-range test to identify significant differences (27). Values in the text are means ± SE.

RESULTS

Growth performance and tissue weight. As shown in Table 2, there was no difference in food intake and growth of rats for 3 wk among the groups. Thus, the feed efficiency also was comparable among the groups (mean values 0.41 to 0.42). Among tissues weighed, there was a tendency of increasing liver weight and decreasing perirenal adipose tissue weight by dietary CLA and the difference between the linoleic acid and 1.0% CLA groups was significant.

Release of chemical mediators from PEC. PEC isolated from rats fed linoleic acid or CLA were incubated with or without calcium ionophore A23187, and the concentrations of histamine and LTB_4 were measured in the medium. The content of histamine in the cells also was measured to estimate the cellular histamine contents. As shown in Figure 1, the effect of CLA on the release of histamine in PEC was diverse, and there was no significant difference in any of the parameters measured. However, the amounts of histamine stored in the cells tended to decrease with an increasing dietary level of CLA. There was a trend toward a reduction in

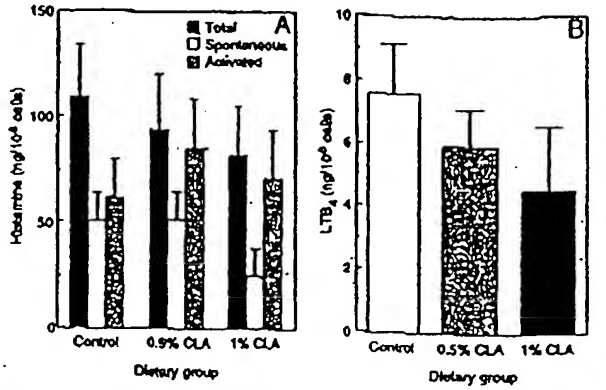

FIG. 1. Effect of dietary conjugated linoleic acid (CLA) on histamine content and release (A) and leukotriene B_4 (LTB_4) release (B) in rat peritoneal exudate cells. Means ± SE of five rats. Histamine release was measured in the presence and absence of calcium ionophore A23187. Total, total amounts of histamine in the cells; Spontaneous, the amount of histamine released during incubation without calcium ionophore A23187; Activated, the amount of histamine which was released from the cells when treated with A23187.

LTB_4 release in response to the dietary level of CLA, but the difference was not significant.

Tissue eicosanoid levels. The effect of CLA on LTB_4 and LTC_4 levels of spleen and lung is shown in Figures 2 and 3, respectively. CLA dose-dependently reduced the level of splenic LTB_4, and the difference between the control and 1% CLA groups was significant. No effect of CLA on the splenic LTC_4 level was observed. However, the concentration of LTC_4 in lung was reduced significantly by CLA even at the 0.5% dietary level. A trend of the dose-dependent reduction of LTB_4 also was observed, but the difference was not significant. The results of the levels of spleen and serum PGE_2 are summarized in Figure 4. CLA significantly reduced the concentration of serum PGE_2, while there was no effect of CLA on the splenic level of PGE_2.

Fatty acid compositions of PEC and splenic lymphocyte lipids. The PUFA composition of PEC and spleen lympho-

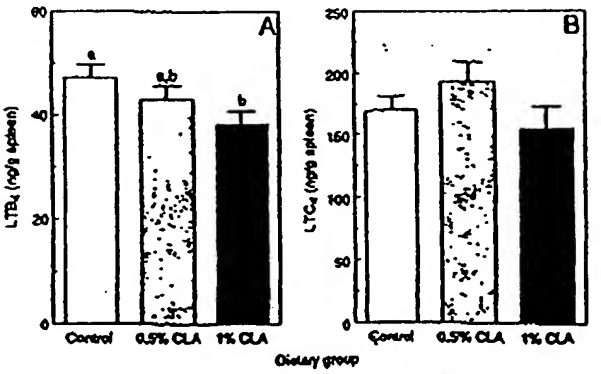

FIG. 2. Effect of dietary CLA on the concentration of splenic (A) LTB_4 and (B) leukotriene C_4 (LTC_4). Mean ± SE of five rats. Values without a common letter are significantly different at $P < 0.05$. For abbreviations see Figure 1.

TABLE 2
Effects of CLA on Growth and Tissue Weights of Rats[a]

Parameter	Group		
	Control	0.5% CLA	1.0% CLA
Initial body weight (g)	102 ± 1	101 ± 1	102 ± 1
Final body weight (g)	170 ± 2	166 ± 3	162 ± 4
Food intake (g/day)	19.1 ± 0.2	18.9 ± 0.3	18.6 ± 0.3
Tissue weight (g/100 g body weight)			
Liver	4.17 ± 0.09[a]	4.11 ± 0.09[a,b]	4.54 ± 0.07[b]
Kidney	0.85 ± 0.03	0.86 ± 0.03	0.87 ± 0.05
Perirenal adipose tissue	1.41 ± 0.07[a]	1.09 ± 0.09[a,b]	0.97 ± 0.14[b]
Heart	0.40 ± 0.02	0.34 ± 0.04	0.34 ± 0.04
Lung	0.48 ± 0.02	0.52 ± 0.02	0.49 ± 0.01
Spleen	0.22 ± 0.01	0.22 ± 0.01	0.25 ± 0.02
Brain	0.66 ± 0.02	0.70 ± 0.01	0.70 ± 0.01
Testis	0.96 ± 0.04	0.87 ± 0.10	1.00 ± 0.03

[a]Mean ± SE of 5 rats. Control group received 1.0% linoleic acid; 0.5% CLA group, 0.5% each of linoleic and CLA; and 1.0% CLA group, 1.0% CLA, respectively. Values without a common superscript letter (a,b) are significantly different at $P < 0.05$. For abbreviation see Table 1.

cyte total lipids is shown in Table 3. There was a dose-dependent reduction by dietary CLA of all n-6 PUFA, 18:2, 20:3, 20:4 and 22:4 in PEC lipids, while there was no difference in the proportion of n-3 PUFA, 22:6 among the groups. A clearer change in these n-6 PUFA was shown in splenic lymphocyte total lipids, and the reduction of 20:4n-6 was significant on a 1.0% CLA diet. Docosahexaenoic acid also tended to decrease with dietary CLA. The decreasing trend of all PUFA in CLA-fed rats was mainly attributable to a moderate increase in major saturated fatty acids, and oleic acid tended to decrease similar to PUFA (data not shown).

Serum thiobarbituric acid value. The concentration of thiobarbituric acid-reactive substance in serum was not modified by dietary CLA, and the values were within 4.1 to 5.5 ng/mL serum in all groups of rats.

Serum Ig levels. As shown in Figure 5, CLA increased the concentration of IgA, IgG and IgM, while decreasing that of IgE in serum. The difference between the control and 1.0% CLA groups was significant in these Ig.

Ig levels in spleen and MLN lymphocytes. Table 4 shows the Ig levels in the medium of rat spleen and MLN lymphocytes cultured for 72 h with or without lipopolysaccharide. Irrespective of the presence or absence of lipopolysaccharide, CLA showed no detectable effects on the Ig levels in spleen lymphocytes except for those of IgM after incubation with lipopolysaccharide, where CLA increased it in a dose-dependent manner. Under the similar situation, CLA increased the concentration of IgA, IgG, and IgM in MLN lymphocytes. The magnitude of the increase was particularly marked at the dietary CLA level of 1.0%. In contrast, there was a significant reduction of the IgE level when the cells from rats fed a 1% CLA diet were incubated with lipopolysaccharide in comparison with the control. A similar response to CLA also was observed even when these cells were incubated for 24 h (data not shown).

Subsets of MLN lymphocytes. The proportion of T-lymphocyte populations of MLN was analyzed as $CD4^+$ and $CD8^+$ subsets. There were no effects of CLA on their relative proportions ($CD4^+/CD8^+$ ratio; 2.6 ± 0.3, 2.4 ± 0.2, and 2.8 ± 0.1 for the control, 0.5% CLA, and 1.0% CLA, respectively).

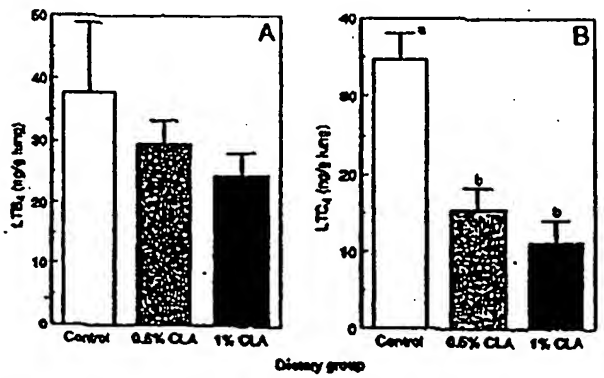

FIG. 3. Effect of dietary CLA on the concentration of lung (A) LTB_4 and (B) LTC_4. Mean ± SE of five rats. Values without a common letter are significantly different at $P < 0.05$. For abbreviations see Figures 1 and 2.

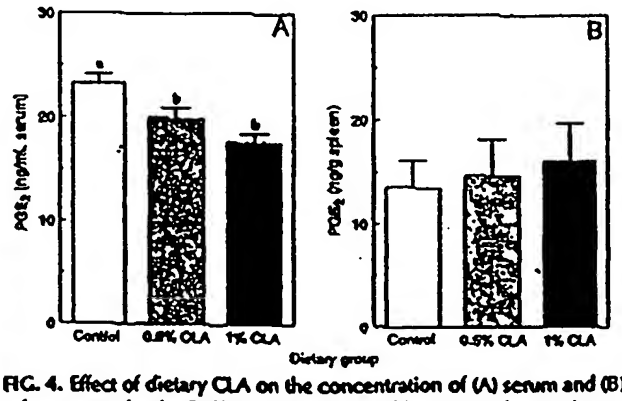

FIG. 4. Effect of dietary CLA on the concentration of (A) serum and (B) spleen prostaglandin E_2 (PGE_2). Mean ± SE of five rats. Values without a common letter are significantly different at $P < 0.05$. For other abbreviation see Figure 1.

TABLE 3
Effects of CLA on Polyunsaturated Fatty Acid Compositions of Peritoneal Exudate Cells and Spleen Lymphocyte Total Lipids of Rats[a]

Cells and fatty acid	Group		
	Control	0.5% CLA	1.0% CLA
		(wt%)	
Peritoneal exudate cells			
18:2n-6	5.5	5.3	4.2
20:3n-6	0.8	0.7	n.d.
20:4n-6	12.7	11.3	9.0
22:4n-6	5.6	5.3	4.2
22:6n-3	0.6	0.6	0.5
CLA			
9t,11c/9c,11t	n.d.	0.1	0.2
10t,12c	n.d.	0.2	0.2
Spleen lymphocytes			
18:2n-6	12.2 ± 0.8	10.4 ± 0.9	9.3 ± 0.9
20:3n-6	1.6 ± 0.2	1.3 ± 0.3	0.9 ± 0.1
20:4n-6	20.2 ± 0.8[a]	15.4 ± 1.3[a,b]	14.7 ± 1.7[b]
22:4n-6	2.5 ± 0.1	2.0 ± 0.2	1.9 ± 0.2
22:6n-3	1.2 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
CLA			
9t,11c/9c,11t	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
10t,12c	n.d.	0.2 ± 0.0	0.2 ± 0.0

[a]Values are means of two pooled samples from two and three rats each for the exudate cells, and means ± SE of three, five, and five rats for control, 0.5% CLA, and 1.0% CLA, respectively. Values without a common superscript letter (a,b) are significantly different at $P < 0.05$; n.d., not detected. For other abbreviation see Table 1.

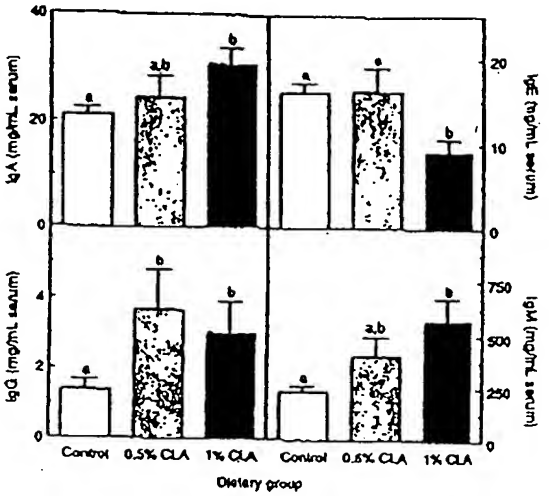

FIG. 5. Effect of dietary CLA on the concentration of serum immunoglobulins (Ig). Mean ± SE of five rats. Values without a common lettter (a,b) are significantly different at $P < 0.05$. For abbreviation see Figure 1.

DISCUSSION

The pathway from linoleate to arachidonate and then eicosanoids is crucial to a range of metabolic diseases (28,29). Food allergy is one such disorder, and it is known that some eicosanoids are involved as chemical mediators in the manifestation of clinical symptoms of hypersensitivity (12,13). The inhibitors of LT production have now been clinically adopted (30,31). However, less is known of the effect that food components exert on this process. Although several food components have been shown to reduce eicosanoid production *in vitro*, in most cases it is practically unsatisfactory because of the limited efficacy (21,22). The results of the present study showed that CLA effectively controlled the production of LTB_4, LTC_4, and PGE_2. CLA significantly reduced LTC_4 production in the lung but not in the spleen. A similar tissue-specific reduction of LTC_4 was observed in rats given sesamin and α-tocopherol simultaneously, while in the spleen LTB_4 but not LTC_4 was reduced (21,22). These observations suggest a complex interaction between dietary fat and antioxidants in the LT-producing system.

Numbers of animal studies showed that dietary PUFA effectively modify the production of eicosanoids, and there is an interaction between n-6 and n-3 PUFA (32). PUFA of the n-3 family suppress the production of eicosanoids from arachidonic acid and exert a substantial suppressing effect on carcinogenesis in breast and colon (33,34). However, the anticarcinogenic effect of n-3 PUFA is far less than that of CLA (2–4). Eicosanoid production is known to be dependent on

TABLE 4
Effects of CLA on the Immunoglobulin Production in Splenic and Mesenteric Lymph Node Lymphocytes of Rats[a]

Immunoglobulin	Without lipopolysaccharide			With lipopolysaccharide		
	Control	0.5% CLA	1% CLA	Control	0.5% CLA	1% CLA
Spleen (ng/mL)						
IgA	3.75 ± 1.23	4.83 ± 0.99	3.78 ± 0.96	9.74 ± 2.45	13.6 ± 3.27	8.30 ± 2.50
IgG	51.0 ± 4.6	53.8 ± 2.3	61.5 ± 2.8	68.1 ± 2.4	71.9 ± 1.9	74.4 ± 1.9
IgM	223 ± 22	228 ± 6	246 ± 9	311 ± 9[A]	348 ± 8[B]	394 ± 6[C]
IgE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Mesenteric lymph node (ng/mL)						
IgA	1.65 ± 0.13[a]	4.78 ± 1.77[b]	5.05 ± 0.10[b]	2.91 ± 0.23[A]	8.72 ± 0.90[B]	22.3 ± 0.7[C]
IgG	n.d.	3.08 ± 0.69[a]	28.1 ± 4.38[b]	n.d.	4.64 ± 0.11[A]	31.9 ± 4.1[B]
IgM	1.86 ± 0.34[a]	4.74 ± 0.50[a]	96.6 ± 13.4[b]	2.85 ± 0.44[A]	6.36 ± 0.48[B]	122 ± 9[C]
IgE	3.81 ± 0.32	4.02 ± 0.33	3.64 ± 0.47	4.81 ± 0.17[A]	4.52 ± 0.29[A]	3.74 ± 0.21[B]

[a]Means ± SE of five rats. Values without a common superscript letter (A,B,C,a,b,c) are significantly different at $P < 0.05$. The lymphocytes were incubated with or without lipopolysaccharide for 72 h, and the concentration of immunoglobulins (Ig) in the supernatant was measured; n.d., not detected.

the substrate availability (35). CLA reduced the proportion of n-6 PUFA including arachidonic acid in the immune cells as observed in the liver and other tissues (8,14). Because of the limited availability of PEC samples for fatty acid analysis, they were analyzed as two pooled samples from two and three rats each. Though the number of analysis may not permit us to draw a definite conclusion, it seems likely that fatty acid composition of PEC also responded similarly as in spleen lymphocytes. This reduction was at least responsible for the reduced production of LT and PG in these cells. CLA may affect metabolic interconversion of fatty acids in the liver that may ultimately result in modified fatty acid composition and arachidonate-derived eicosanoid production in extrahepatic tissue (14). However, more direct participation of the metabolites of CLA cannot be ruled out (9,36). Therefore, the present study added possible usefulness of CLA for controlling the allergic reaction caused by food. Since the effect of CLA on Ig production differed between MLN lymphocytes and spleen lymphocytes, the analysis of the fatty acid composition of the former cells may provide a clue to understanding the mechanism of action.

In contrast to the eicosanoid production, the level of histamine released from PEC, which reflects the mast cell degranulation by a receptor-independent pathway, apparently was not modified by CLA and more directly the fatty acid composition of membrane phospholipids. Engels *et al.* (37) observed that the type of dietary fats and thus the change in the fatty acid composition of mast cell phospholipids did not influence the cell degranulation process. CLA is reported to be incorporated into triglyceride more preferably than phospholipids in tumor cells (7). Thus, CLA may not substantially influence the fatty acid composition of membrane phospholipids and hence, the structure and function of the membrane. In such a situation, the degranulation of the mast cells may not be modified largely.

An interesting observation is that CLA regulates the Ig production class specifically. Food allergy reaction is initiated by the production of allergen-specific IgE (12,13). IgA, in contrast, serves as an antiallergic factor by interfering with the intestinal absorption of allergen, and IgG also works as an antiallergic factor through the competition with binding of allergen to the receptor on the surface of the target cells such as mast cells and basophiles (12,13). CLA increased the production of IgA and IgG, while reducing that of IgE in lymphocytes, in particular MLN lymphocytes irrespective of the presence or absence of lipopolysaccharide, a cell activator. The response of splenic lymphocytes to CLA was less clear except for a slight but significant increase in IgM after lipopolysaccharide activation. However, the response pattern similar to MLN lymphocytes was observed in serum, indicating that CLA can modify the Ig levels preferably even on a whole-body basis. Bile acids (24) and unsaturated fatty acids (25) also regulate antibody production class specifically, but in a manner contrasted from that of CLA. These compounds may promote the allergic response through an increase in IgE production and a reduction in IgA and IgG production. It is plausible that the production of IgE and of IgA and IgG are at least reciprocally regulated. Thus, in addition to the favorable effect on the eicosanoid production, CLA was expected to mitigate the food allergic reaction.

The amounts of CLA ingested by rats of the present study corresponded to approximately 30 and 60 mg/100 g body weight for 0.5 and 1.0% CLA diets, respectively. These amounts were pharmacological when extrapolated to human, 18 and 36 g/60 kg body weight/day. However, as in the case of weight reduction in man, approximately 3 g/d for 2 to 3 mon, a prolonged ingestion may produce a favorable effect even at a lower dose. A long-term trial with a lower dietary level of CLA merits further study.

In conclusion, CLA produced a situation favorable for mitigation of food allergic reaction. Since the effect was seen at a dietary level as low as 0.5 or 1.0%, it is likely that CLA can strongly regulate multiple metabolic processes. Thus, the clinical application of CLA is warranted. Studies with immunized animals will provide more direct information regarding this issue.

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REFERENCES

1. Ip, C., Scimeca, J.A., and Thompson, H.J. (1994) Conjugated Linoleic Acid. A Powerful Anticarcinogen from Animal Fat Sources, *Cancer 74*, 1050–1054.
2. Belury, M.A. (1995) Conjugated Dienoic Linoleate: A Polyunsaturated Fatty Acid with Unique Chemoprotective Properties, *Nutr. Rev. 53*, 83–89.
3. Hanmann, B.F. (1996) Conjugated Linoleic Acid Offers Research Promise, *INFORM 7*, 152–159.
4. Doyle, E. (1998) Scientific Forum Explores CLA Knowledge, *INFORM 9*, 69–72.
5. Ip, C. (1996) Multiple Mechanisms of Conjugated Linoleic Acid in Mammary Cancer Prevention, in *Breast Cancer—Advances in Biology and Therapeutics* (Calvo, F., Crepin, M., and Magdelenat, H., eds.) pp. 53–58, John Libbey Eurotext Ltd., Montrouge, France.
6. Ip, C., and Scimeca, J.A. (1997) Conjugated Linoleic Acid and Linoleic Acid Are Distinctive Modulators of Mammary Carcinogenesis, *Nutr. Cancer 27*, 131–135.
7. Ip, C., Jiang, C., Thompson, H.J., and Scimeca, J.A. (1997) Retention of Conjugated Linoleic Acid in the Mammary Gland Is Associated with Tumor Inhibition During the Post-Initiation Phase of Carcinogenesis, *Carcinogenesis 18*, 755–759.
8. Sugano, M., Tsujita, A., Yamasaki, M., Yamada, K., Ikeda, I., and Kritchevsky, D. (1997) Lymphatic Recovery, Tissue Distribution, and Metabolic Effects of Conjugated Linoleic Acid in Rats, *J. Nutr. Biochem. 8*, 38–43.
9. Sebedio, J.L., Juaneda, P., Dobson, G., Ramilison, L., Martin, J.D., and Chardigny, J.M. (1997) Metabolites of Conjugated Isomers of Linoleic Acid (CLA) in the Rat, *Biochim. Biophys. Acta 1345*, 5–10.
10. Calder, P.C. (1995) Fatty Acids, Dietary Lipids and Lymphocyte Functions, *Biochem. Soc. Trans. 23*, 302–309.

11. Zurier, R.B. (1993) Fatty Acids, Inflammation and Immune Responses, *Prostaglandins Leukotrienes Essent. Fatty Acids 48*, 57–62.
12. Metcalfe, D.D. (1991) Food Allergy, *Curr. Opinion Immunol. 3*, 881–886.
13. Lemke, P.J., and Taylor, S.L. (1994) Allergic Reactions and Food Intolerances, in *Nutritional Toxicology* (Kotsonis, F.N., Mackey, H., and Hjelle, J., eds.) pp. 117–137, Raven Press, Ltd., New York.
14. Belury, M.A., and Kempa-Steczko, A. (1997) Conjugated Linoleic Acid Modulates Hepatic Lipid Composition in Mice, *Lipids 32*, 199–204.
15. Wong, M.W., Chew, B.P., Wong, T.S., Hosick, H.L., Boylston, T.D., and Shultz, T.D. (1997) Effects of Dietary Conjugated Linoleic Acid on Lymphocyte Function and Growth of Mammary Tumors in Mice, *Anticancer Res. 17*, 987–993.
16. Ip, C., Chin, A.F., Scimeca, J.A., and Pariza, M.W. (1991) Mammary Cancer Prevention by Conjugated Dienoic Derivative of Linoleic Acid, *Cancer Res. 69*, 6118–6124.
17. Ha, Y.L., Grimm, N.K., and Pariza, M.W. (1989) Newly Recognized Anticarcinogenic Fatty Acids: Identification and Quantification in Natural and Processed Cheeses, *J. Agric. Food Chem. 37*, 75–81.
18. Reeves, P.G., Nielsen, F.H., and Fahey, G.C. (1993) AIN-93 Purified Diets for Laboratory Rodents: Final Report of the American Institute of Nutrition *Ad Hoc* Writing Committee on the Reformulation of the AIN-76A Rodent Diet, *J. Nutr. 123*, 1939–1951.
19. Matsuo, N., Yamada, K., Yamashita, K., Shoji, K., Mori, M., and Sugano, M. (1995) Inhibitory Effect of Tea Polyphenols on Histamine and Leukotriene B_4 Release from Rat Peritoneal Exudate Cells, *In Vitro Cell. Develop. Biol. 32*, 340–344.
20. Powell, W.S. (1987) Precolumn Extraction and Reversed-Phase High-Pressure Liquid Chromatography of Prostaglandins and Leukotrienes, *Anal. Biochem. 164*, 117–131.
1. Gu, J.-Y., Nonaka, M., Yamada, K., Yoshimura, K., Takasugi, M., Ito, Y., and Sugano, M. (1994) Effect of Sesamin and α-Tocopherol on the Production of Chemical Mediators and Immunoglobulins in Brown-Norway Rats, *Biosci. Biotech. Biochem. 58*, 1855–1858.
22. Gu, J.-Y., Wakizono, Y., Tsujita, A., Lim, B.O., Nonaka, M., Yamada, K., and Sugano, M. (1995) Effect of Sesamin and α-Tocopherol, Individually or in Combination, on the Polyunsaturated Fatty Acid Metabolism, Chemical Mediator Production, and Immunoglobulin Levels in Sprague-Dawley Rat, *Biosci. Biotech. Biochem. 59*, 2198–2202.
23. Shore, P.A., Burkhalter, A., and Cohn, V. (1959) A Method for the Fluorometric Assay of Histamine in Tissues, *J. Pharmacol. Exp. Ther. 127*, 182–186.
24. Lim, B.O., Yamada, K., and Sugano, M. (1994) Effect of Bile Acids and Lectins on Immunoglobulin Production in Rat Mesenteric Lymph Node Lymphocytes, *In Vitro Cell. Dev. Biol. 30A*, 407–413.
25. Yamada, K., Hung, P., Yoshimura, K., Taniguchi, S., Lim, B. O., and Sugano, M. (1996) Effect of Unsaturated Fatty Acids and Antioxidants on Immunoglobulin Production by Mesenteric Lymph Node Lymphocytes of Sprague-Dawley Rats, *J. Biochem. 120*, 138–144.
26. Lim, B.O., Yamada, K., Nonaka, M., Kuramoto, Y., Hung, P., and Sugano, M. (1997) Dietary Fibers Modulate Indices of Intestinal Immune Function in Rats, *J. Nutr. 127*, 663–667.
27. Duncan, D.B. (1995) Multiple Range and Multiple F Test, *Biometrics 11*, 1–42.
28. Kinsella, J.E., Lokesh, B., Broughton, S., and Whelan, J. (1990) Dietary Polyunsaturated Fatty Acids and Eicosanoids: Potential Effects on the Modulation of Inflammatory and Immune Cells: An Overview, *Nutrition 6*, 24–44.
29. Holman, R.T. (1997) ω3 and ω6 Essential Fatty Acid Status in Human Health and Disease, in *Handbook of Essential Fatty Acid Biology* (Yehuda, S., and Mostofsky, D.I., eds.) pp. 139–182, Humana Press, Totowa, NJ.
30. Harris, R.A., Cater, G.W., Bell, R.L., Moore, J.L., and Brooks, D.W. (1995) Clinical Activity of Leukotriene Inhibitors, *Int. J. Immunopharmacol. 17*, 147–156.
31. Ara, G., and Teicher, B.A. (1996) Cyclooxygenase and Lipoxygenase Inhibitors in Cancer Therapy, *Prostaglandins Leukotrienes Essent. Fatty Acids 54*, 3–16.
32. Simopoulos, A.P. (1996) Metabolic Effect of Omega-3 Fatty Acids and Essentiality, in *Handbook of Lipids in Human Nutrition* (Spiller, G.A., ed.) pp. 51–73, CRC Press, Inc., New York.
33. Carroll, K.K. (1991) Dietary Fat and Cancer, *Am. J. Clin. Nutr. 53*, 1064S–1067S.
34. Glauert, H.P. (1992) Dietary Fatty Acids and Cancer, in *Fatty Acids in Foods and Their Health Implications* (Chow, C.K., ed.) pp. 753–768, Marcel Dekker, Inc., New York.
35. Lee, H.L., Ikeda, I., and Sugano, M. (1992) Effects of Dietary n-6/n-3 Polyunsaturated Fatty Acid Balance on Tissue Lipid Levels, Fatty Acid Patterns, and Eicosanoid Production in Rats, *Nutrition 8*, 162–166.
36. Ip, C., Briggs, S.P., Haegele, A.D., Thompson, H.J., Storkson, J., and Scimeca, J.A. (1996) The Efficacy of Conjugated Linoleic Acid in Mammary Cancer Prevention Is Independent of the Level or Type of Fat in the Diet, *Carcinogenesis 17*, 1045–1050.
37. Engels, W., VanHaaster, C.M.C.J., Lemmens, P.J.M.R., VanderVusse, G.J., and Horustra, G. (1997) Dietary Modulation of Fatty Acid Composition of Mast Cell Phospholipids Does Not Affect Histamine Release Induced by Compound 48/80, *Inflammation Res. 46*, 185–190.

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X. RELATED PROCEEDINGS APPENDIX

A copy of the Decision On Appeal mailed July 20, 2005, for Appeal No. 2005-0150 for U.S. Patent Application Serial Nos. 09/271,024, filed March 17, 1999, is provided with the present appeal brief.

A copy of the Decision On Appeal mailed August 30, 2005, for Appeal No. 2005-1578 for U.S. Patent Application Serial Nos. 09/132,593, filed August 11, 1998, is provided with the present appeal brief.

APPENDIX 2

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Ex parte ASGEIR SAEBO, and CARL SKARIE

Appeal No. 2005-1578
Application No. 09/132,593

ON BRIEF

Before WILLIAM F. SMITH, ADAMS, and GRIMES, Administrative Patent Judges.

ADAMS, Administrative Patent Judge.

DECISION ON APPEAL

This is a decision on the appeal under 35 U.S.C. § 134 from the examiner's final rejection of claims 1-6 and 8, which are all the claims pending in the application.

Claim 1 is illustrative of the subject matter on appeal and is reproduced below:

1. A food product comprising conjugated linoleic acid alkyl esters in a biologically active concentration, said alkyl esters comprising less than about two percent trans,trans; 8,10 and 11,13 octadecadienoic acid isomers.

The references relied upon by the examiner are:

Baltes et al. (Baltes)	3,162,658	Dec. 22, 1964

Cook et al. (Cook)	5,554,646	Sep. 10, 1996
Cain et al. (Cain)	WO 97/18320	May 22, 1997

Chin et al. (Chin), "Dietary Sources of Conjugated Dienoic Isomers of Linoleic Acid, a Newly Recognized Class of Anticarcinogens," J. Food Composition And Analysis, Vol. 5, pp. 185-197 (1992)

GROUND OF REJECTION

Claims 1-6 and 8 stand rejected under 35 U.S.C. § 103 as being unpatentable over the combination of Cook, Cain, Chin and Baltes.

We reverse.

DISCUSSION

According to the examiner (Answer, page 3), Cook "teach an active form of conjugated linoleic acid, i.e., 10,12-octadecadienoic acid and 9,11-octadecadienoic acid, which includes esters, salts and free acids of conjugated linoleic acid." In addition, the examiner finds (Answer, page 4), Cook teach that "[t]he conjugated linoleic acid may be obtained through isomerization of safflower oil;" "a food product comprising said active form of conjugated linoleic acid;" and that "[c]9, t11- and t10, c12-isomer[s] are the predominantly major isomers of the conjugated linoleic acid active form...." According to the examiner Cook do not teach 8,10- and 11,13-octadecadienoic acid isomers. Id. Therefore, the examiner reasons (id.), since Cook does not mention the 8,10- and 11,13-octadecadienoic acid isomers they must not be present and therefore, Cook meets appellants' claimed requirement of less than 2 percent 8,10- and 11,13-octadecadienoic acid isomers.

Regarding Chin and Cain, the examiner finds (id.), Chin "teach that it is known that c9;t11-conjugated linoleic acid isomer is an active form of conjugated linoleic acid," and that Cain "teaches a CLA [(conjugated linoleic acid)] composition made from sunflower oil for food additive contains 48.9% of c9, t11, 51.1% of t10,c12 linoleic acid or their esters."

Based on this evidence the examiner concludes (Answer, page 5),

> it would have been prima facie obvious to a person of ordinary skill in the art, at the time the claimed ... invention was made, to make a conjugated linoleic alkyl ester mixture from sunflower oil or safflower oil comprising c9, t11- and t10, c12-octadecadienoic moieties without/or with less than 2% of 8,10- and 1,13-octadecadienoic ester, such as those disclosed by Cain et al., and employ the mixture in food products.

In response, appellants argue (Brief, page 6), the Sæbo Declaration establishes that the compositions of Cook and Cain "cannot produce alkyl esters comprising less than about two percent trans,trans; 8,10 and 11,13 octadecadienoic acid isomers." According to the Sæbo Declaration (received December 9, 2004), repeat experiments were performed using the methodology described in Cook and Cain. For Cook, the Sæbo Declaration reports (paragraph 4),

> this conjugation method resulted in in [sic] a conjugated linoleic acid composition comprising approximately 1.58% c11,t13 CLA and 2.34% t9,t11 and t10,t12 CLA. The t8,c10 isomer co-elutes with the c9,t11 isomers, but almost always occurs in a one to one proportion to the c11,t13 isomer.

Accordingly, the trans,trans isomers resulting from Cook's conjugation method are outside the requirements of appellants' claimed invention, which requires, inter alia, less than two percent trans, trans isomers.

Regarding Cain, the Sæbo Declaration reports (paragraph 6)

> this conjugation method resulted in a conjugated linoleic acid composition comprising approximately 3.49% c11,t13 CLA and 2.24% t9,t11 and t10,t12 CLA. The t8,c10 isomer co-elutes with the c9,t11 isomers, but almost always occurs in a one to one proportion to the c11,t13 isomer.

Accordingly, the trans,trans isomers resulting from Cain's conjugation method are outside the requirements of appellants' claimed invention, which requires, inter alia, less than two percent trans, trans isomers.

In response, the examiner asserts (Answer, page 6), "the declaration fails to establish the fact that the conjugated linoleic acid disclosed by Cook or Cain as recited in the prior office action contains more than 2% of the isomers identified in claim 1 herein." In support of this assertion, the examiner finds (Answer, bridging paragraph, pages 6-7), while Cain acknowledges the existence of trans,trans isomers, Cain "do not disclose the presence of trans isomers in their CLA composition." Apparently, the examiner is of the opinion that since Cain and Cook do not specifically state that their CLA compositions contain isomers other than t10,c12- and c9,t11-octadecadienoic acid, the CLA compositions taught by Cain and Cook only contain t10,c12- and c9,t11-octadecadienoic acid. We are not persuaded by the examiner's assertion.

According to Cook (column 1, lines 65 to column 2, line 3);

> [I]n one preferred embodiment of the method of the present invention the safe and effective amount of conjugated linoleic acid, which is selected from 9,11-octadecadienoic acid; 10,12-octadecadienoic acid; mixtures thereof; and non-toxic salts thereof is added to the feed of an animal in which it is desired to reduce the body fat.

We note, however, that according to Cook (column 4, lines 22-24, emphasis added), "[t]he terms 'conjugated linoleic acids' and 'CLA' as used herein are intended to include 9,11-octadecadienoic acid, [and] 10,12-octadecadienoic acid...." Thus, while Cook emphasizes the 9,11- and 10,12-octadecadienoic acid isomers, Cook leaves his definition of CLA open to "include" other isomers. In addition, Cook does not distinguish which geometric isomer is intended by the recitation of 9,11-octadecadienoic acid and 10,12-octadecadienoic acid. In this regard, we note that there is no requirement in Cook's claims that a particular CLA, let alone a particular geometric isomer of 9,11- or 10,12-octadecadienoic acid is required. Further, while the examiner recognizes (Answer, page 4), Cook discloses that "[c]9,t11- and t10,c12-isomer[s] are the predominantly major isomers of the conjugated linoleic acid...", the examiner fails to appreciate that Cook discloses (column 4, lines 48-50), "8 possible geometric isomers of 9,11 and 10,12-octadecadienoic acid (c9,c11; c9,t11; t9,c11; t9,t11; c10,c12; c10,t12; t10,c12 and t10,t12)...", all of which fall within Cook's definition of CLA. Accordingly, we fail to understand how the examiner has read Cook's disclosure as limited to a composition containing only the c9,t11- and t10,c12-isomers of octadecadienoic acid.

According to Cook (column 4, lines 28-29), "[t]he preferred method of synthesizing CLA is that described in Example 1", which appears in Column 2 of Cook. According to the Sæbo Declaration, in the repeat of Cook, "the conjugation conditions were the same as those described in [c]olumn 2 of ... [Cook]." The results reported in the Sæbo Declaration are consistent with Cook in that a CLA composition was obtained that included the 9,11 and 10,12 isomers of octadecadienoic acid. Cf. Cook, column 4, lines 22-24, emphasis added), "[t]he terms 'conjugated linoleic acids' and 'CLA' as used herein are intended to include 9,11-octadecadienoic acid, [and] 10,12-octadecadienoic acid...." While the results reported in the Sæbo Declaration are consistent with the disclosure of Cook, they are inconsistent with the requirements of appellants' claimed invention, because they include more than 2% of the trans,trans octadecadienoic acid isomer. Specifically, the resulting CLA composition contains, inter alia, 2.34% t9,t11 and t10,t12 CLA. For the foregoing reasons we are not persuaded by the examiner's assertions regarding Cook.

Regarding Cain, the reference discloses (page 3), "our invention concerns a new process for the preparation of CLA's, wherein the ratio $\frac{cis^{9}\text{-}trans^{11}}{trans^{10}\text{-}cis^{12}}$ can be chosen freely." Therefore, contrary to the examiner's assertion (Answer, page 7), it is not unreasonable for Cain to not report on the presence of other isomers in his CLA compositions, isomers other than cis^{9}-$trans^{11}$ and $trans^{10}$-cis^{12} were simply not the focus of his invention. Cf. Sæbo Declaration, paragraph 7, "Cain may have simply chosen not to include non-

active isomers when reporting their results." In this regard, we note that Cain state (page 5), "our invention also concerns novel organic materials, ... wherein the conjugated polyunsaturated fatty acid moieties at least comprise two isomers L_1 and L_2 ...." According to Cain (id.), "is it preferred that L_1 and L_2 are cis^9 $trans^{11}$ and $trans^{10}$ cis^{12}-linoleic acid (or vice versa)[.]" See also, for example, claims 1, 6 and 9 of Cain, wherein similar language is used.

Therefore, similar to the facts in Cook, while Cain emphasizes the cis^9 $trans^{11}$ and $trans^{10}$ cis^{12} isomers, Cain's compositions may comprise other CLA isomers. Accordingly, we see nothing inconsistent with the results of the repeat of Cain's methodology as presented in the Sæbo Declaration. Paragraph 6 of the Sæbo Declaration, and the results attached at Tab 2 of the Declaration, reports that Cains' methodology results in a composition comprising at least two isomers, the cis^9 $trans^{11}$ and $trans^{10}$ cis^{12} isomers. The results also demonstrate however, that other isomers are also present in the resulting composition. Specifically, the resulting CLA composition contains, inter alia, 2.24% t9,t11 and t10,t12 CLA. For the foregoing reasons we are not persuaded by the examiner's assertions regarding Cain.

On reflection, we disagree with the examiner's conclusion (Answer, page 5), that it would have been prima facie obvious to a person of ordinary skill in the art, at the time the invention was made to combine the teachings of Cain, Cook and Chin[1] in the manner necessary to arrive at appellants' claimed invention.

[1] In our opinion, the examiner's reliance (Answer, page 4) on Chin to teach that c9,t11-conjugated linoleic acid isomer is an active form of conjugated linoleic acid, is insufficient to make up for the deficiency in the combination of Cain and Cook.

We also note the examiner's reliance on Baltes (Answer, page 5), to "teach that employment of low alkali alcoholate as catalysts for isomerization of unconjugated polyethenoid fatty acid compounds to conjugated isomers is known." However, in our opinion, Baltes fails to make up for the deficiency in the combination of Cain and Cook.

Prima facie obviousness based on a combination of references requires that the prior art provide "a reason, suggestion, or motivation to lead an inventor to combine those references." Pro-Mold and Tool Co. v. Great Lakes Plastics Inc., 75 F.3d 1568, 1573, 37 USPQ2d 1626, 1629 (Fed. Cir. 1996).

> [E]vidence of a suggestion, teaching, or motivation to combine may flow from the prior art references themselves, the knowledge of one of ordinary skill in the art, or, in some cases, from the nature of the problem to be solved. . . . The range of sources available, however, does not diminish the requirement for actual evidence. That is, the showing must be clear and particular.

In re Dembiczak, 175 F.3d 994, 999, 50 USPQ2d 1614, 1617 (Fed. Cir. 1999) (citations omitted). The suggestion to combine prior art references must come from the cited references, not from the application's disclosure. See In re Dow Chemical Co., 837 F.2d 469, 473, 5 USPQ2d 1529, 1531 (Fed. Cir. 1988). Based on the foregoing, it is our opinion that the examiner failed to meet his burden of presenting the evidence necessary to support a prima facie case of obviousness. If the examiner fails to establish a prima facie case, the rejection is improper and will be overturned. In re Fine, 837 F.2d 1071, 1074, 5 USPQ2d 1596, 1598 (Fed. Cir. 1988).

Accordingly, we reverse the rejection of claims 1-6 and 8 under 35 U.S.C. § 103 as being unpatentable over the combination of Cook, Cain, Chin and Baltes.

REVERSED

William F. Smith
Administrative Patent Judge

Donald E. Adams
Administrative Patent Judge

Eric Grimes
Administrative Patent Judge

) BOARD OF PATENT
) APPEALS AND
) INTERFERENCES

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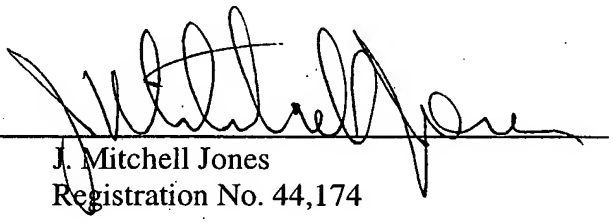
XI. CONCLUSION

For the foregoing reasons, it is submitted that the Office's rejection of Claims 1-10 was erroneous, and reversal of the rejection is respectfully requested. Appellant requests either that the Board render a decision as to the allowability of the claims, or alternatively, that the application be remanded for reconsideration by the Office.

Dated: January 23, 2006



J. Mitchell Jones
Registration No. 44,174

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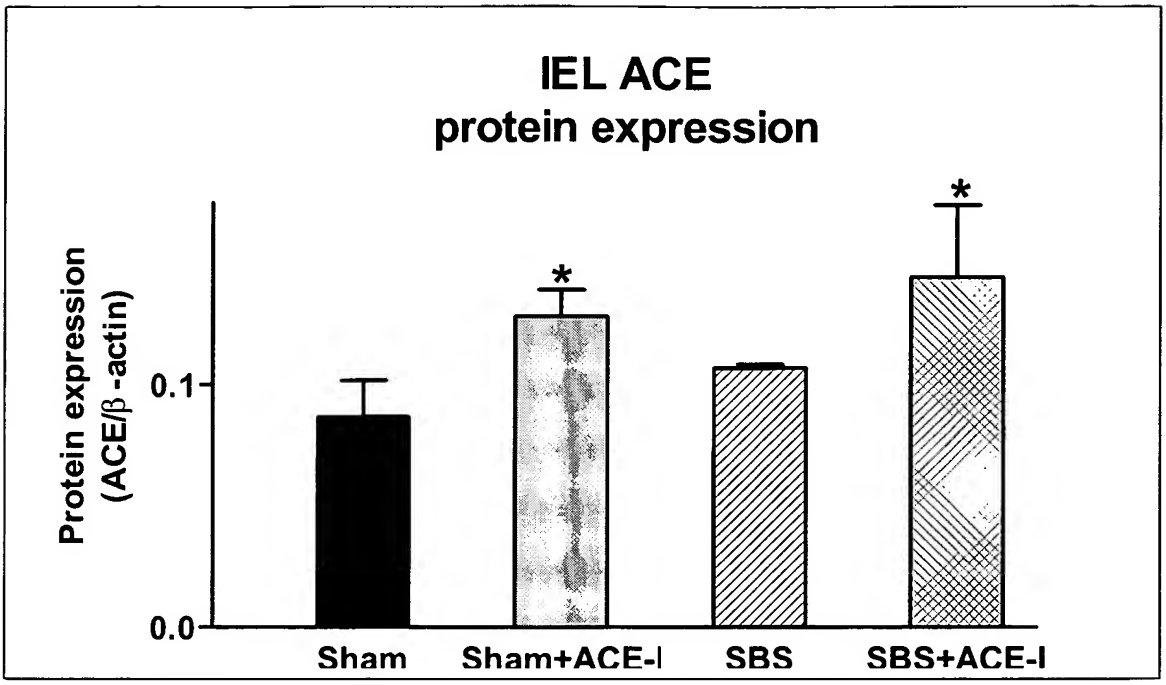
San Francisco, CA 94105
608/218-6900

FIGURE 1


IEL ACE
protein expression
Protein expression
(ACE/β-actin)
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0.0
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Sham
Sham+ACE-I
SBS
SBS+ACE-I

FIGURE 2

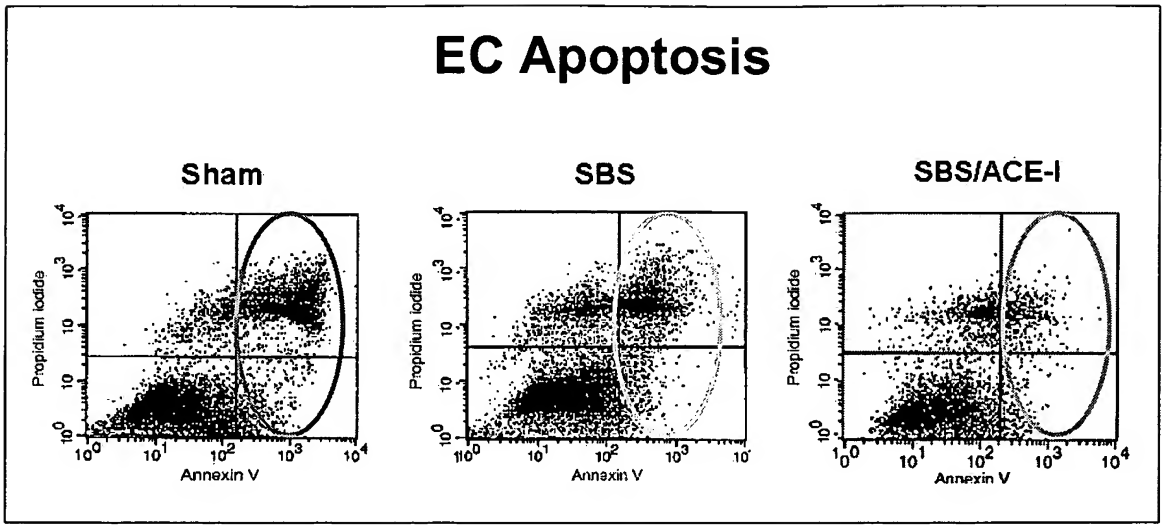


FIGURE 3

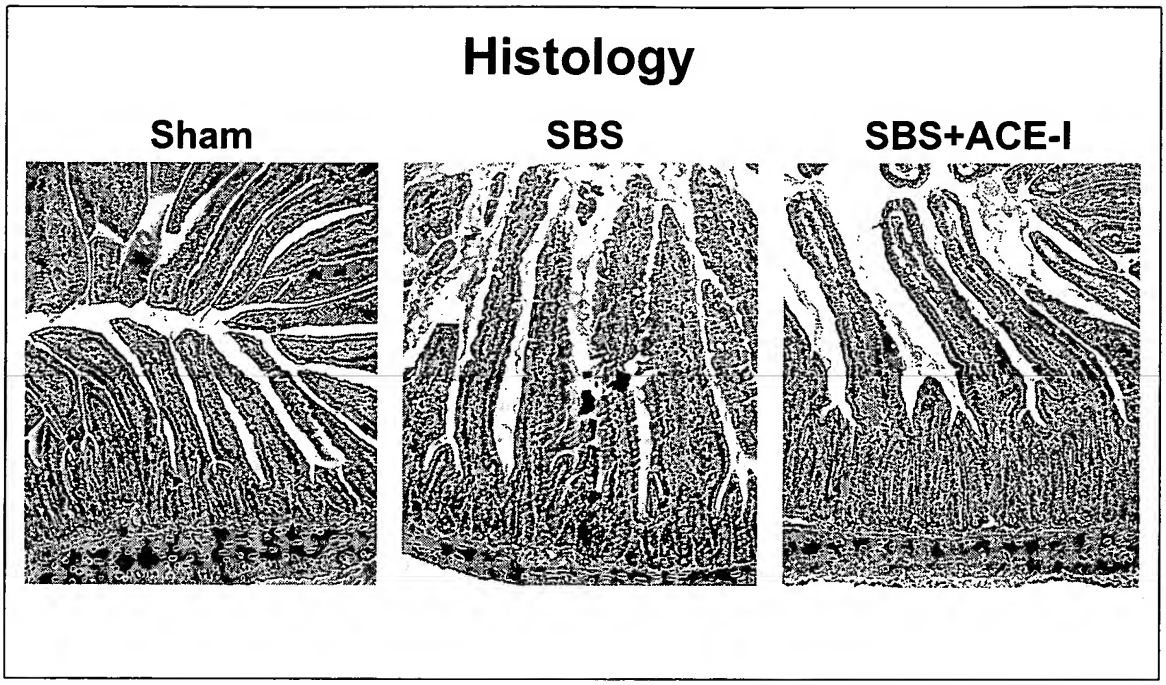
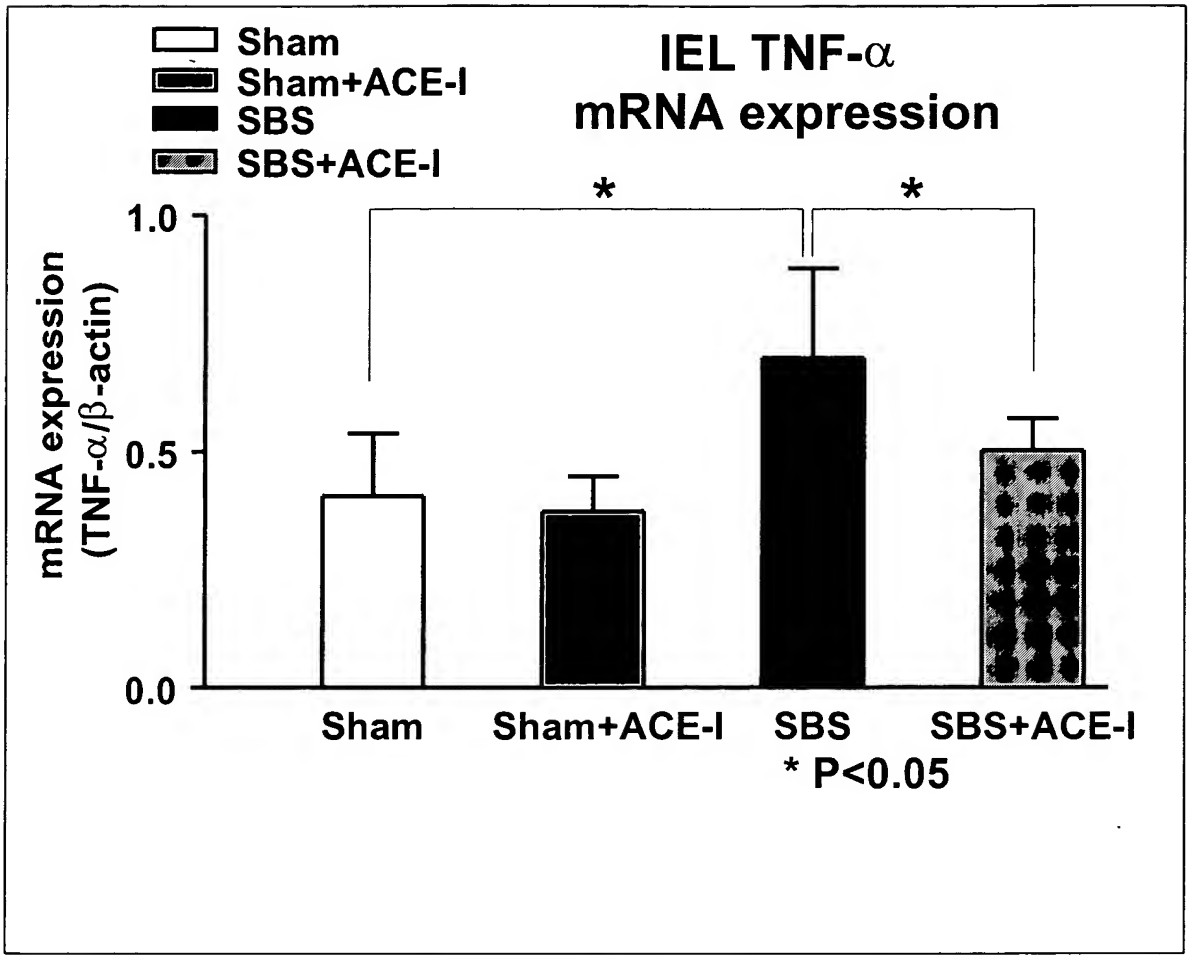


FIGURE 4


Sham
Sham+ACE-I
SBS
SBS+ACE-I
IEL TNF-α
mRNA expression
*
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mRNA expression
(TNF-α/β-actin)
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0.5
0.0
Sham
Sham+ACE-I
SBS
SBS+ACE-I
* P<0.05

FIGURE 5

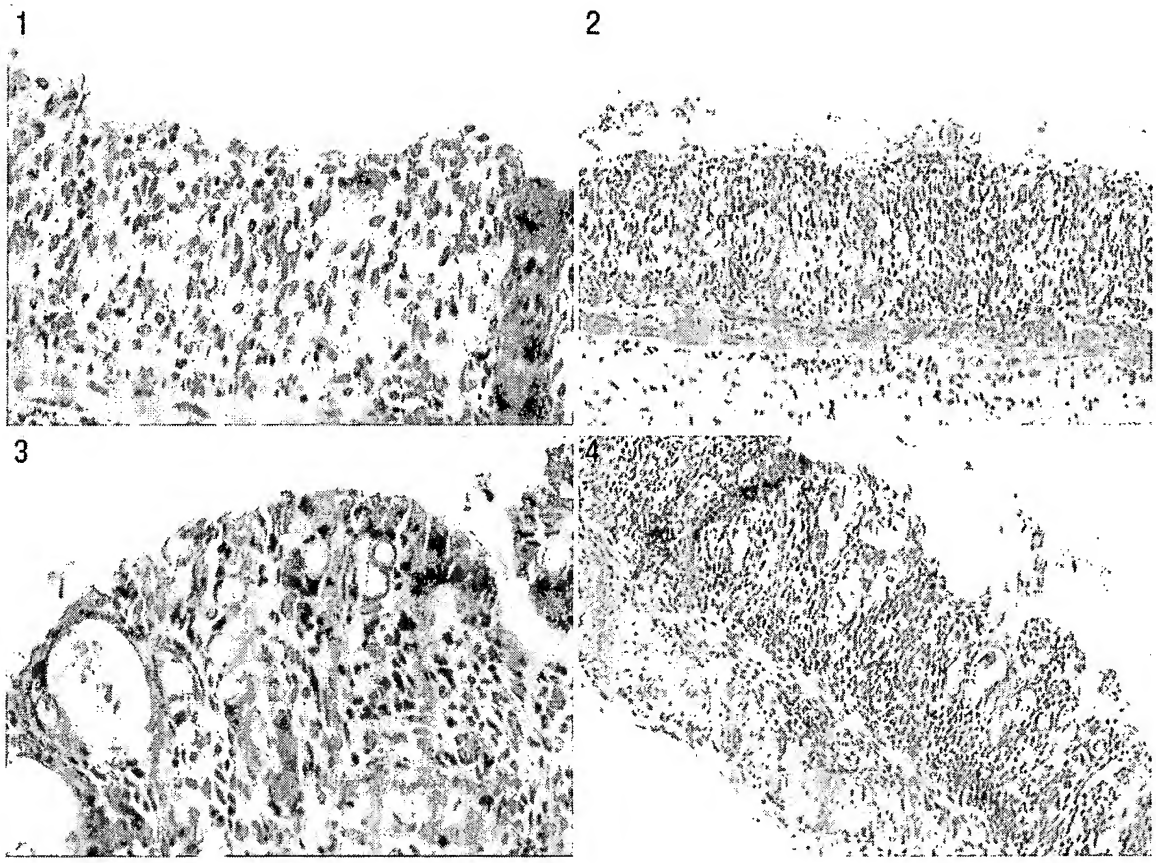
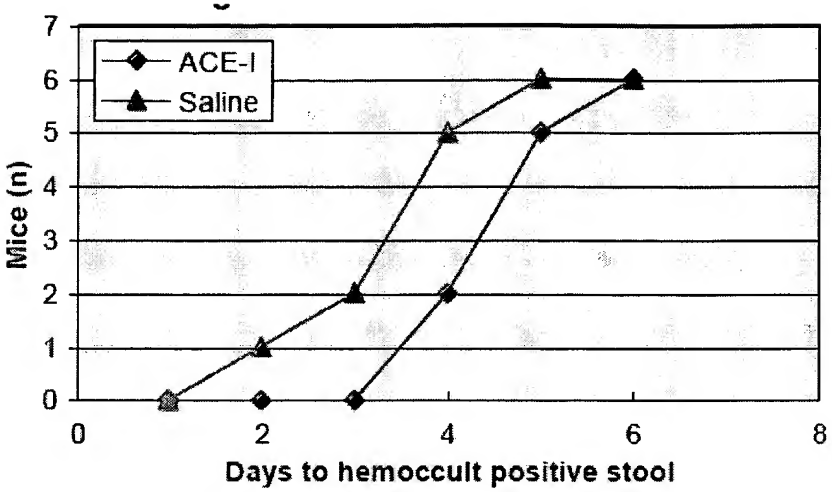


FIGURE 6


ACE-I
Saline
Mice (n)
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1
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2
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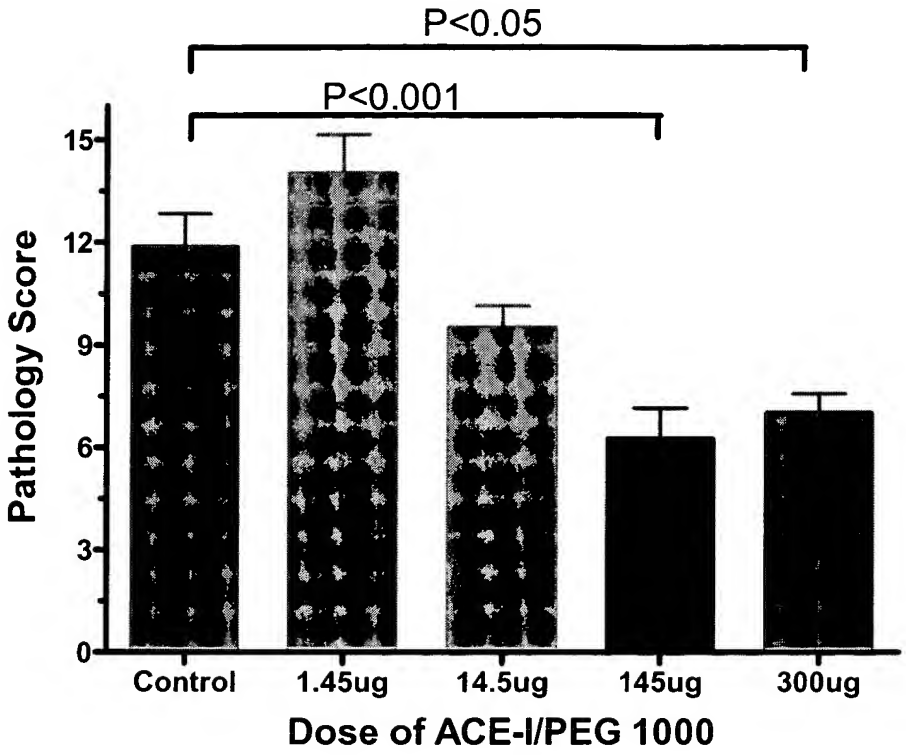
Days to hemoccult positive stool

FIGURE 7


P<0.05
P<0.001
Pathology Score
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12
9
6
3
0
Control
1.45ug
14.5ug
145ug
300ug
Dose of ACE-I/PEG 1000

APPENDIX 3

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Ex parte ASGEIR SAEBO, CARL SKARIE, DARIA JEROME, and GUDMUNDER HAROLDSSON

Appeal No. 2005-0150
Application No. 09/271,024

HEARD: June 7, 2005

Before WILLIAM F. SMITH, ADAMS and GRIMES, Administrative Patent Judges.

ADAMS, Administrative Patent Judge.

DECISION ON APPEAL

This is a decision on the appeal under 35 U.S.C. § 134 from the examiner's final rejection of claims 5-8 and 13-17, which are all the claims pending in the application.[1]

Claims 5 and 13 are illustrative of the subject matter on appeal and are reproduced below:

5. A biologically active acylglycerol composition comprising a plurality of acylglycerol molecules wherein the acylglycerol molecules comprise substituents R1, R2, and R3 attached at the positions of the OH-

[1] While the examiner states (Answer, page 2), "[t]he statement of the status of the claims contained in the brief is correct," we note that appellants' Brief does not address the status of claim 12. For clarity, we note that appellants cancelled claim 12, along with claims 1-4 and 9-11 in the amendment (see page 1) received November 14, 2000.

groups of a glycerol backbone, and wherein R1, R2 and R3 are selected from the group consisting of a hydroxyl group and an octadecadienoic acid, said composition characterized in containing at least approximately 30% t10,c12 octadecadienoic acid, at least approximately 30% c9,t11 octadecadienoic acid, and about less than 1% total of 8,10 octadecadienoic acid, 11,13 octadecadienoic acid and trans-trans octadecadienoic acid at positions R1, R2 and R3, wherein said percentages are peak area percentages as determined by gas chromatography.

13. A composition comprising a prepared food product containing a biologically active acylglycerol composition comprising a plurality of acylglycerol molecules wherein the acylglycerol molecules comprise substituents R1, R2, and R3 attached at the positions of the OH-groups of a glycerol backbone, and wherein R1, R2 and R3 are selected from the group consisting of a hydroxyl group and an octadecadienoic acid, said composition characterized in containing at least approximately 30% t10,c12 octadecadienoic acid, at least approximately 30% c9,t11 octadecadienoic acid, and about less than 1% total of 8,10 octadecadienoic acid, 11,13 octadecadienoic acid and trans-trans octadecadienoic acid at positions R1, R2 and R3, wherein said percentages are peak area percentages as determined by gas chromatography.

The references relied upon by the examiner are:

Pariza et al. (Pariza)	5,017,614	May 21, 1991
Nilsen et al. (Nilsen)	5,885,594	Mar. 23, 1999
Cain et al. (Cain)	WO 97/18320	May 22, 1997

GROUNDS OF REJECTION

Claims 5-8 stand rejected under 35 U.S.C. 102(a) as anticipated by Cain.

Claims 13-17 stand rejected under 35 U.S.C. § 103 as being unpatentable over Cain.

Claims 5-8 and 13-17 stand rejected under 35 U.S.C. § 103 as being unpatentable over Nilsen in view of Cain and Pariza.

We reverse.

DISCUSSION

According to the examiner (Answer, page 3), the basis for each rejection is "fully set forth in prior office action, paper No. 26, mailed March 26, 2003." However, upon inspection of the Office Action mailed March 26, 2003 (see page 2), we find that instead of providing a statement of the rejection, the examiner refers to the "reasons set forth in the prior office action." It is in the Office Action mailed August 13, 2002 where we find a statement of each rejection on this record. We remind the examiner, as set forth in § 1208(A) of the Manual of Patent Examining Procedure

> Examiners may incorporate in the answer their statement of the grounds of rejection merely by reference to the final rejection (or a single other action on which it is based, MPEP § 706.07). Only those statements of grounds of rejection appearing in a single prior action may be incorporated by reference. An examiner's answer should not refer, either directly or indirectly, to more than one prior Office action. Statements of grounds of rejection appearing in actions other than the aforementioned single prior action should be quoted in the answer.

THE REJECTION UNDER 35 U.S.C. § 102:

According to the examiner (page 3, Office Action, mailed August 13, 2002),

> Cain teaches [example 6] an acyglycerol composition comprising mono-[,] di-[,] and tri-glyceride[s] wherein the fatty acid[s] are c9,t11 CLA[2] or t10, c12 CLA, wherein the total CLA in the composition is about ... [61.9%], of which 48.9% was the cis 9, trans 11 isomer and 51.1% was the trans 10, cis 12 isomer. No other CLA isomers are indicated, or suggested to be present in the composition.

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[2] According to Cain (page 1), CLA refers to compositions containing free conjugated linoleic acid. Cf. appellants' specification (page 9), "[a]s used herein, 'conjugated linoleic acid' or 'CLA' refers to any conjugated linoleic acid or octadecadienoic free fatty acid."

"Under 35 U.S.C. § 102, every limitation of a claim must identically appear in a single prior art reference for it to anticipate the claim." Gechter v. Davidson, 116 F.3d 1454, 1457, 43 USPQ2d 1030, 1032 (Fed. Cir. 1997). "Every element of the claimed invention must be literally present, arranged as in the claim." Richardson v. Suzuki Motor Co., Ltd., 868 F.2d 1226, 1236, 9 USPQ2d 1913, 1920 (Fed. Cir. 1989). Upon review of Cain we agree with the examiner that example 6 of Cain teaches a composition comprising "61.9% of conjugated linoleic acid (CLA) of which 48.9% was the cis 9, trans 11 isomer and 51.1% was the trans 10, cis 12 isomer." In addition, we agree with the examiner that Cain is silent regarding the presence of other CLA isomers that may be present in the composition. Thus, the composition taught by Cain appears, in the first instance, to meet all the limitations of appellants' claimed invention. Accordingly, we find that the examiner has established a sufficient evidentiary basis to shift the burden to appellants to demonstrate that Cain does not anticipate their claimed invention. In re Spada, 911 F.2d 705, 708, 15 USPQ2d 1655, 1658 (Fed. Cir. 1990) ("when the PTO shows sound basis for believing that the products of the applicant and the prior art are the same, the applicant has the burden of showing that they are not."). In re King, 801 F.2d 1324, 1327, 231 USPQ 136, 138 (Fed. Cir. 1986); In re Ludtke, 441 F.2d 660, 664, 169 USPQ 563, 566 (CCPA 1971).

In response, appellants assert (Brief, page 5), Cain "does not anticipate [c]laim[s] 5-8 because the methods utilized by Cain et al. cannot produce the claimed CLA isomer profile (i.e., a CLA composition containing less than 1%

total of 8,10 octadecadienoic acid, 11,13 octadecadienoic acid and trans-trans octadecadienoic acid isomers)." In support of this assertion, appellants direct our attention to the Saebo Declaration, which according to appellants "establishes that the compositions of Cain et al. necessarily include the 8,10 and 11,13 isomers of CLA." According to the Saebo Declaration (paragraph 4),

> In the repeat of Cain, the conjugation conditions were the same as those described in Example 6 of WO97/18320. The results of the conjugation reactions were analyzed by GC-MS. ... [T]his conjugation method resulted in a conjugated linoleic acid composition comprising approximately 3.49% c11,t13 CLA and 2.24% t9,t11 and t10,t12 CLA. The t8,c10 isomer co-elutes with the c9,t11 isomers, but almost always occurs in a one to one proportion to the c11,t13 isomer.

From this, appellants assert (Brief, page 8), "[a]pplicants followed the exact instructions of Cain and analyzed the product. The [a]pplicants did not fail to obtain CLA. Indeed, they obtained CLA with the isomers described by Cain et al. However, the fact remains that the CLA also contained other isomers that are not mentioned by Cain." According to appellants (Brief, bridging paragraph, pages 8-9), Cain's "silence concerning the presence of the isomers cannot be equated with the absence of the isomers. ... [Cain] does not specifically define CLA to include non-active CLA isomers." On this point the Saebo Declaration states (paragraph 5),

> [t]he [e]xaminer states ... that Cain teaches CLA compositions that are composed of 48.9% c9,t11 and 51.1% t10,c12 CLA, and that the analysis was carried out with gas chromatography and no other isomer of conjugated linoleic acid is detected. However, this does not mean that the other isomers were not present, as was found in my repeat of Cain. This discrepancy is explainable by the facts that 1) methods for the analysis of CLA compositions in 1996 were rather crude and 2)

> Cain may have simply chosen not to include non-active isomers when reporting their results.

In addition, appellants direct our attention to Sugano[3]. Brief, bridging paragraph, pages 10-11.[4] According to appellants (id.), Sugano "isomerized linoleic acid [under] conditions similar to those described by Cain...." However, as appellants explain (id.), in contrast to the results reported by Cain, Sugano's "resulting CLA preparation contained the following CLA isomers: 29.8% c9,t11/t9,c12; 1.3% c9,c11; 1.4%c10, c12; 18.6% t9,t11/t10,t12; 5.6% linoleic acid; and 13.7% other isomers." In view of the foregoing, appellants assert (Brief, page 11), "[i]n contrast to the simplified analysis presented in Cain et al., isomerization of CLA results in the production of many different isomers, not just the desired c9,t11 and t10,c12 isomers."

Appellants also direct out attention (Brief, page 11), to examples 1-4 of their specification in further support of their position that the methodology taught by Cain would have resulted in the production of CLA compositions that do not meet the limitations of their claimed invention. According to appellants (id., emphasis removed),

> [t]hese examples compare non-aqueous alkali isomerization under high or low temperatures and aqueous alkali isomerization under high or low temperatures. The important fact to note is that

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[3] Sugano et al. (Sugano), "Conjugated Linoleic Acid Modulates Tissue Levels of Chemical Mediators and Immunoglobulins in Rats," Lipids, Vol. 33, No. 5, pp. 521-527 (1998).

[4] Appellants also direct out attention to "Chapter 5 of the book Advances in Conjugated Linoleic Acid Research, Volume 2, J. Sebedio, W.W. Christie, and R. Adolf, Eds., AOCS Press, Champaign, IL, 2002...." See Brief, bridging sentence, pages 9-10. This reference, however, was published after appellants' March 17, 1999 filing date. Publications dated after the filing date providing information publicly first disclosed after the filing date generally cannot be used to show what was known at the time of filing. See In re Gunn, 537 F.2d 1123, 1128, 190 USPQ 402, 405 (CCPA 1976). Accordingly, we have not considered this reference.

> each reaction, even the low temperature non-aqueous alkali isomerization reaction (Example 1, Table 6), produced a distribution of the expected isomers, not just the c9,t11 and t10,c12 isomers.

From this appellants assert (id., emphasis removed), "the compositions of Cain necessarily contained levels [of] 8,10; 11,13; and trans,trans isomers that do not meet the[ir] claimed levels."

In response, the examiner appears to back away from his original finding (page 3, Office Action, mailed August 13, 2002) that "[n]o other CLA isomers are indicated, or suggested to be present in the composition" taught by Cain. In response to appellants' arguments, and contrary to his original inference, the examiner asserts (Answer, page 4), "nowhere in Cain states that 'conjugated linoleic acid' are exclusively for c9, t11; and t10, c12 isomers." Thus, the examiner appears to concede that the CLA compositions taught by Cain would be expected to contain additional CLA isomers other than the c9, t11; and t10, c12 isomers identified by Cain.

The examiner maintains, however, "there is no convincing evidence showing that Cain's composition has the amount of the particular isomers herein claimed." Apparently the examiner is referring to the requirement of appellants' claimed invention that the acylglycerol composition comprise "about less than 1% total of 8,10 octadecadienoic acid, 11,13 octadecadienoic acid and trans-trans octadecadienoic acid at positions R_1, R_2 and R_3 ...." While the examiner appreciates that the composition taught by Cain would contain CLA isomers other than t10,c12 and c9,t11 octadecadienoic acid, the examiner makes no

attempt to explain why the compositions taught by Cain would necessarily contain "less than 1% total of 8,10 octadecadienoic acid, 11,13 octadecadienoic acid and trans-trans octadecadienoic acid at positions R_1, R_2 and R_3 ..." as required by appellants' claimed invention. The only evidence on this record that addresses this point is appellants'. As discussed above, both the Saebo Declaration (using the same methodology as set forth in Cain), and the Sugano reference (using a similar methodology as set forth in Cain), resulted in a CLA composition that contained more than "about less than 1% total of 8,10 octadecadienoic acid, 11,13 octadecadienoic acid and trans-trans octadecadienoic acid at positions R_1, R_2 and R_3 ...." In our opinion, the evidence of record weighs in favor of appellants, and rebuts the examiner's prima facie case of anticipation.

Accordingly, we reverse the rejection of claims 5-8 under 35 U.S.C. § 102(a) as anticipated by Cain.

THE REJECTIONS UNDER 35 U.S.C. § 103:

Cain:

According to the examiner (page 3, Office Action, mailed August 13, 2002), "Cain teaches an acylglycerol composition comprising mono-[,] di-[,] and tri-glyceride[s] wherein the fatty acids are c9,t11 CLA or t10, c12 CLA, no other CLA isomers are indicated, or suggested to be present in the composition. See, example[s] 6-10 at page[s] 16-22." The examiner finds that Cain characterize

> all the fatty acid[s] through gas chromatography and ... identified the CLA. For example, in example 6, ... [Cain] state[s] "[t]he fatty

> acid composition of the product, as determined by FAME GC, contained 63.8% CLA, of which 48.9% was the cis 9, trans 11 isomer and 51.1% was the trans 10, cis 12 isomer." See page 16, lines 17-21.

From this the examiner asserts (id.), "the rest of the fatty acids are not CLA, and the CLA is composed entirely of cis 9, trans 11[ ]isomer and trans 10, cis 12 isomer."

In addition, the examiner finds (Answer, bridging paragraph, pages 3-4) that Cain teaches the use of the acylglycerol composition "in various food products including ice cream, soup, and bakery products. See, particularly, examples 12-17 at page 24-35 and the claims." The examiner recognizes, however, that Cain does not teach "that each of the isomers must be 30% or more of the total CLA moieties for the particular food products." Answer, page 4. Nevertheless, the examiner asserts (id.),

> it would be obvious to employ such [a] CLA composition in the food product, since such [a] CLA composition [comprising 48.9% was the cis 9, trans 11 isomer and 51.1% was the trans 10, cis 12 isomer] has been expressly disclosed by Cain [for use in a food product]. See, ... example 6.

In response, appellants assert (Brief, page 12), "[a]s established above [with regard to the rejection under 35 U.S.C. 102(a)], the compositions of Cain necessarily contain levels [of] 8,10; 11,13; and trans,trans isomers that do not meet the claimed levels. Thus, Cain et al. does not render the claims obvious." Similarly, the examiner relies on his response to the anticipation rejection. See Answer, page 6.

Accordingly, for the reasons set forth above, we find that the evidence of record weighs in favor of appellants. Therefore, the rejection of claims 13-17 under 35 U.S.C. § 103 as being unpatentable over Cain is reversed.

Nilsen in view of Cain and Pariza:

According to the examiner (page 4, Office Action, mailed August 13, 2002), Nilsen "teach a composition comprising 90-100[]% of an acylglycerol compound wherein the fatty acid radical is a conjugated polyunsaturated fatty acid." In this regard, the examiner finds (id.), "[t]he preferred conjugated polyunsaturated fatty acid is conjugated linoleic acid which is defined as c9, t11-octadecadienoic acid and/or c10, t12-octadecadienoic acid." The examiner recognizes, however, that Nilsen does not teach "the employment of the combination of c9, t11-octadecadienoic acid and/or t10, c12-octadecadienoic acid in the acylglycerol, or the specific amounts of each of the two isomers...."

The examiner relies on Cain to make up for Nilsen's deficiency regarding the specific c9, t11, and t10, c12 isomers of octadecadienoic acid in the acylglycerol taught by Nilsen. According to the examiner (page 5, Office Action, mailed August 13, 2002), Cain "teach[es] that both c9, t11-octadecadienoic acid and t10, c12-octadecadienoic acid are considered the active isomers of CLA, and are known to be beneficial for animal health." In this regard, the examiner relies on Pariza (id.), "to show that [a] person of ordinary skill in the art possess the skill of preparing/or isolating the pure single isomer employed herein. See,

particularly, column 4, line 50, bridging column 8, lines 68, wherein, the separation, purification, and analysis of the isomers are discussed."

To make up for Nilsen's failure to teach an acylglycerol composition containing at least approximately 30% c9, t11-octadecadienoic acid and t10, c12-octadecadienoic acid, the examiner asserts (id.), "[t]he optimization of the ratio of the compounds is considered within the skill of the artisan."

Based on this evidence, the examiner finds (id.),

> it would have been prima facie obvious to a person of ordinary skill in the art, at the time the claimed ... invention was made, to make the composition of Nilsen et al. with acyglycerol [sic] compounds wherein the fatty acid moiety is a mixture of about equal amounts of c9, t11-octadecadienoic acid and t10, c12-octadecadienoic acid and employ the composition in feed for animals.

In this regard, the examiner asserts (id.), Nilsen did "not use ... other isomers of conjugated linoleic acids.... Therefore[, Nilsen] meet[s] the limitation set forth in claim 5 that other isomers are present in amounts less than 1%...."

In response, appellants assert (Brief, page 13), Cain "does not teach compositions comprising less than 1% 8,10; 11,13; and trans-trans isomers or methods of obtaining such compositions." Regarding Nilsen, appellants assert (id., emphasis removed), like Cain, Nilsen "provides no such compositions or methods [nor does Nilsen] teach any method at all for conjugation, they merely list CLA in a long list of fatty acids that may be useful in their invention." In support of this assertion, appellants rely on paragraph 6 of the Saebo Declaration which states "[w]ith respect to the Nilsen reference, I note that it does not provide any method of producing conjugated linoleic acid having less

than 1% 8,10; 11,13; and trans-trans isomers." Regarding Pariza, appellants assert (Brief, bridging paragraph, pages 13-14), "does not teach preparation of CLA in amounts suitable for incorporation into acylglycerides. Indeed, the HPLC purified isomers are produced for use as chromatography standards. Importantly, because the isomers are produced for use as standards, Pariza does not teach or suggest combining the isomers to form a composition containing both t10,c12 and t9,c11 isomers are required by the [c]laims." See also Saebo Declaration, paragraph 7. Accordingly, appellants assert (Brief, page 14), Pariza "teaches away from a combination of isomers as required by the [c]laims."

In response, the examiner addresses each reference individually. Accordingly, we will address the examiner's discussion of each reference in turn.

Cain:

The examiner relies (Answer, page 8) on his response to the anticipation rejection to address appellants' assertions regarding Cain. Accordingly, for the reasons set forth above, we are not persuaded by the examiner's assertion.

Nilsen:

Regarding Nilsen, the examiner asserts (id.), "one of ordinary skill in the art would have been expected to be able to practice the invention claimed by Nielsen [sic], including making an acylglycerol compound wherein the Rs are conjugated linoleic acids (specifically defined as c9, t11; t10, c12 isomers), see the claims in Nielsen [sic] et al." We fail to see the relevance of the examiner's reference to the claims of Nilsen. Upon consideration of Nilsen's claimed

invention we find no specific reference to c9, t11; t10, c12 isomers of CLA. At best, Nilsen's claims relate to a genus of CLA isomers. In this regard, we note the examiner's reference (Answer, page 6, emphasis added), to column 4, lines 4-6 of Nilsen, for what the examiner believes to be Nilsen's disclosure of "[t]he preferred conjugated polyunsaturated fatty acid ... which is defined as c9, t11-octadecadienoic acid and/or c10, t12-octadecadienoic acid." Appellants' claimed invention is directed to, inter alia, an acylglycerol composition containing at least approximately 30% t10, c12 octadecadienoic acid, not c10, t12-octadecadienoic acid. The examiner identifies no section of Nilsen, and we find none, that would suggest appellants' specific acylglycerol composition. Further, the examiner offers to response to appellants' assertion that Nilsen provides no method through which to produce an acylglycerol composition as set forth in appellants' claimed invention. Accordingly, we are not persuaded by the examiner's assertions to the contrary.

Pariza:

In response to appellants' argument concerning Pariza, the examiner asserts (Answer, page 8), "[a]ppellants concede[] that Paris [sic] et al. does provide purified CLA isomers, but nevertheless argue that Pariza's disclosure is for producing standard samples for HPLC, and is not in a scale suitable for making acylglycerol herein claimed." To this the examiner asserts (id.), "there is no limitation as to the quantity of the composition in claims 5-8." On reflection, we are not persuaded by the examiner's assertions.

While appellants do not dispute that Pariza teaches methods of making t10, c12 and c9, t11 octadecadienoic acid, appellants assert (Brief, page 13), "Pariza does not teach preparation of CLA in amounts suitable for incorporation into acylglycerides. Indeed, the HPLC purified isomers are produced for use as chromatography standards." In response, the examiner does not dispute that amount of t10, c12 and c9, t11 octadecadienoic acid produced in the method of Pariza would not be sufficient to produce appellants' claimed acylglycerol composition. Instead, the examiner concludes (Answer, page 8), "preparative HPLC would be obvious to one of ordinary skill in the art with similar condition[s]." Apparently, it is the examiner's position that a person of ordinary skill in the art would have found it obvious to scale-up the method taught by Pariza to produce a sufficient amount of t10, c12 and c9, t11 octadecadienoic acid to incorporate into acylglycerol molecules. The evidence of record, however, does not support the examiner's assertion. Further, the examiner fails to provide any evidence that the method taught by Pariza could be effectively scaled-up to produce the acylglycerol molecules required by appellants' claimed invention. In the absence of a reasonable expectation of success one is left with only an "obvious to try" situation which is not the standard of obviousness under 35 U.S.C. § 103. See In re O'Farrell, 858 F.2d 894, 904, 7 USPQ2d 1673, 1681 (Fed. Cir. 1988).

In order to establish a prima facie case of obviousness, there must be more than the demonstrated existence of all of the components of the claimed subject matter. There must be some reason, suggestion, or motivation found in

the prior art whereby a person of ordinary skill in the field of the invention would make the substitutions required. That knowledge cannot come from the applicants' disclosure of the invention itself. Diversitech Corp. v. Century Steps, Inc., 850 F.2d 675, 678-79, 7 USPQ2d 1315, 1318 (Fed. Cir. 1988); In re Geiger, 815 F.2d 686, 688, 2 USPQ2d 1276, 1278 (Fed. Cir. 1987); Interconnect Planning Corp. v. Feil, 774 F.2d 1132, 1143, 227 USPQ 543, 551 (Fed. Cir. 1985). On the record before us, we find no reasonable suggestion for combining the teachings of the references relied upon by the examiner in a manner which would have reasonably led one of ordinary skill in this art to arrive at the claimed invention. The initial burden of presenting a prima facie case of obviousness rests on the examiner. In re Oetiker, 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992). In our opinion, the examiner has failed to provide the evidence necessary to support a prima facie case of obviousness.

Accordingly, we reverse the rejection of claims 5-8 and 13-17 under 35 U.S.C. § 103 as being unpatentable over Nilsen in view of Cain and Pariza.

REVERSED

William F. Smith
Administrative Patent Judge

Donald E. Adams
Administrative Patent Judge

Eric Grimes
Administrative Patent Judge

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